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COMPLETE SPECIFICATION

NEW SEQUENCE OF M. PARATUBERCULOSIS, AND ITS USE FOR THE CLONING AND EXPRESSION OF NUCLEOTIDE SEQUENCES

We, INSTITUT PASTEUR, a body established and existing under the laws of France, of 25-28, rue du Dr. Roux, 75724 Paris Cedex 15, France; and MASSEY UNIVERSITY, a body corporate established under the Massey University Act 1963, of Palmerston North, New Zealand,

hereby declare the invention for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:-

The present invention relates to a nucleotide sequence enabling the cloning or expression of nucleotide sequences in a specific host-cell.

A nucleotide sequence according to the invention can be obtained from Mycobacterium paratuberculosis.

Among mycobacteria, certain strains are particularly well known, for example the Calmette and Guerin bacillus (BCG), which is a non-virulent strain of Mycobacterium bovis widely used throughout the world for vaccination against tuberculosis. Its biological characteristics make it a worthwhile candidate for the development of recombinant vaccines. The cell wall functions as a very effective adjuvant and a single inoculation can give rise to long-lasting immunity. Serious side effects due to this bacillus are rare even with repeated immunisations.

The induction of specific immunity following vaccination with BCG is initiated when the T cells interact with macrophages presenting the antigens of mycobacteria in association with the products of the major histocompatability complex (abbreviation MHC). The clones of sensitized T cells proliferate and produce lymphokines which in turn activate the macrophages to eliminate the bacilli in a non-specific manner. In addition, helper T cells induce the proliferation of clones of B cells, leading to the production of antibodies.

Attempts have already been made to effect the cloning and expression of heterologous genes in BCG, particularly by utilizing the knowledge available regarding replicative or integrative vectors. Thus an epitope of the gag protein of HIV-1 has been cloned in the form of a polypeptide fusion with the antigen α, this antigen being one of the major proteins secreted by mycobacteria, particularly BCG or Mycobacterium Kansasii, and antibiotic resistance genes have been expressed under the control of their own regulatory regions.

In order to optimize the expression of heterologous antigens in recombinant BCG, the inventors have oriented their research towards determining the characteristics of the

functional gene-regulation units in mycobacteria. From this research, the inventors have isolated and characterized nucleotide sequences from Mycobacterium paratuberculosis enabling the expression of given nucleic acids in the cells of mycobacteria or other hosts.

By nucleic acid is meant any nucleotide sequence capable of being cloned and/or expressed, whatever its composition, length, or origin (i.e. whether obtained by isolation or synthesis).

Various studies have been carried out in relation to M. paratuberculosis (also referred to hereinafter as Mptb), and Green et al (Nucleic Acids Research, Vol. 17 (22) 1989, pages 9063-9072) have in particular characterized and sequenced an insertion element of this mycobacteria; this element has been called IS900. According to Green et al, this insertion element contains an open reading frame called ORF1197, which codes for a protein of 399 amino acids.

The inventors have investigated specific sequences of the species Mycobacterium paratuberculosis, screening a lambda gt11 gene library and carrying out hybridization tests with the DNA of other mycobacterial strains, in particular M. phlei, described in A. Murray et al, New Zealand Veterinary Journal 37: 47-50. On this occasion they were interested in a specific DNA sequence which contained a fragment adjacent to the IS900 element described by Green et al.

They determined the presence of a sequence adjacent to the 5' end of the inverse sequence complementary to the open reading frame which codes for a potential transposase and is contained on the insertion element IS900; this new sequence is capable of functioning as a promoter and contains important signals for the regulation of transcription and translation.

A nucleotide sequence according to the invention which can be used for the cloning and/or expression of a nucleic acid is characterized in that it comprises a sequence (I) selected from:

a) the sequence shown in Figure 2, or any part of this sequence, capable of being involved in the expression of a nucleic acid, which is placed under its control;

b) a sequence which hybridizes with the sequence complementary to sequence a).

A part of sequence I which is of interest in terms of the present invention can be determined by performing a test such as the following: a given part of sequence I is cloned upstream of a translation signal and promoter recognition vector, containing for example the β-galactosidase gene without its promoter and its first eight amino acids.

The conditions for carrying out this test can be established on the basis of the description corresponding to the constructions in Figures 7 to 9. A part of sequence I, tested in this manner, and of interest in terms of the invention, leads to the synthesis of a protein having a β-galactosidase activity in cells of different hosts, e.g. the actinomycetes.

One nucleotide sequence, which is particularly advantageous in terms of the invention, and which can be used for the cloning and/or expression of a nucleic acid, is sequence (II), characterized in that it comprises:

a) the following nucleotide sequence or any part thereof containing in particular the transcription initiation site (position +1), and elements necessary for the recognition and binding of the RNA polymerases of a particular host cell which will be transformed by this sequence, these elements comprising, in particular, the sequences [TAC ACT] at position -10 relative to the initiation site for transcription and [TC GAC A] at position -35 relative to said site:

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GAT CCC GTG ACA AGG CCG AAG AGC CCG CGA CCG TGC GGT CGT

CGA CGA CCG AGT GTG AGC AGA CCC CCT GGT GAA GGG TGA ATC

GAC AGG TAC ACA CAG CCG CCA TAC ACT TCG CTT CAT GCC CTT (+1 above CAT)

ACG GGG GGC GGC CAA CCC AGA AGG AGA TTC TCA

b) a sequence which hybridizes with the sequence complementary to sequence a).

The hybridization conditions can be described as follows:

One uses, for instance, a probe determined on the basis of the sequence with which it is desired to test the hybridization, labelled with ^{32}P (10^6 cpm/ml). It is placed in contact with the test sequence for 16 hours at 65°C in a hybridization solution (formamide, 50%; 5 x SSPE; salmon sperm DNA, 200 μg/ml; and 10 x Denhart). The membranes on which the hybridization is carried out are then washed twice for 30 minutes in a solution of 1SSC, 0.1% SDS, at ambient temperature (20°C), and then washed twice with 0.1SSC, 0.1% SDS for 30 minutes at 65°C.

<u>Composition of 5 x SSPE</u>

NaCl	900 mM
NaH_2PO_4	450 mM
Na_2EDTA	5 mM
pH	7.4

<u>Denhart</u>

ficoll	2.5 g/l
polyvinyl pyrrolidone	2.5 g/l
BSA (Pentex fraction V)	2.5 g/l

<u>0.1 x SSC</u>

NaCl	15 mM
Na_3 citrate	0.1%

Nucleotide A marked +1 corresponds to a transcription initiation site determined according to the invention, and the nucleotide sequence upstream of this site comprises recognition

and binding elements for RNA polymerases of host cells (regions -35 and -10) in which the sequence is able to be used as a promoter for the cloning or expression of specific nucleic acid sequences. Regions -35 and -10 are located with reference to site +1.

The above sequences (sequences I and II) contain the minimum sequence necessary for the initiation of the transcription defined above, and also fragments upstream and downstream of this sequence which are capable of playing a part in the regulation of the transcription and/or expression. For example, these sequences comprise a sequence of the Shine Dalgarno type [A AGG AG] which is involved in the binding of the ribosome.

Another nucleotide sequence capable of enabling the expression, in a host cell, of a nucleic acid is characterized, according to the invention, in that it comprises a DNA sequence (III) selected from:

a) the following nucleotide sequence:

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                                                 +1
TC GAC AGG TAC ACA CAG CCG CCA TAC ACT TCG CTT CA
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b) a sequence which hybridizes with the sequence complementary to sequence a);

c) any part of this sequence that is involved in the transcription of a nucleic acid.

The sequence designated as b), defined by its ability to hybridize with I, II, or III, gives in each case the variants of the sequence of the present invention which, while being modified in terms of one or more nucleotides, nevertheless retain the properties of sequences I, II, or III according to the invention in the transcription of a nucleic acid. The modifications that can be introduced in the variants are, for example, substitutions, insertions, deletions, or inversions of nucleotides.

The nucleotide sequence comprising one the sequences I, II, or III or a variant of I, II, or III, contains a nucleotide sequence capable of functioning as a promoter for the expression of given sequences of nucleic acids.

The nucleotide sequences of the invention can also in future be designated by the expression "sequence containing the promoter" when they contain sequence III.

If applicable, sequence III can also be used with a fragment of sequence I that is not necessarily adjacent to it in sequence I but which is capable of being associated with sequence I for the expression of a particular nucleic acid.

Said sequences can be obtained by extraction, purification from the DNA of M. paratuberculosis, or by chemical synthesis.

According to another method of implementing the invention, any variant of a nucleotide sequence comprising the above-described sequences I, II, or III can be defined by the fact that it retains the functional properties of sequences I, II, or III and particularly their ability to perform the functions of a promoter in the transcription of nucleic acids in a given host.

Those elements of nucleotide sequence I or sequence II that adjoin sequence III can be deleted at least in part and possibly substituted. For example, the sequence comprised between nucleotide positions +24 and +41 relative to the transcription initiation site can be replaced in whole or in part with a sequence that is exogenous with respect to the sequence naturally present downstream of sequence III in Mptb, this exogenous sequence comprising a Shine Dalgarno sequence recognizable by the ribosome in a given host.

By way of example, this sequence comprised between positions +2 and +41 can be replaced with an exogenous sequence of bacterial origin, for example from E. coli, comprising a Shine Dalgarno sequence.

The invention also relates to the use of any part of sequence I or II, outside of regions -35 and -10 or the Shine Dalgarno (SD) sequence, that is capable of being involved in the transcription or translation of a given nucleic acid.

The invention also relates to a recombinant nucleotide sequence characterized in that it comprises a nucleotide sequence as defined above and at least one nucleic acid sequence whose cloning and/or expression is desired in a given host cell, under the control of this nucleotide sequence.

This nucleic acid sequence can be a sequence that codes for an Mptb peptide, or a heterologous sequence that codes for a peptide or polypeptide of different origin.

A nucleic acid sequence is considered as being a heterologous sequence when it does not correspond to the sequence naturally adjacent to sequence I or II in Mptb; part of this naturally adjacent sequence corresponds to the sequence of 716 bp represented in Figure 2.

The various elements of the invention can thus be used for the expression, in a host cell other than an Mptb host cell, of a nucleic acid sequence that codes for a peptide or polypeptide of Mptb, under the control of the nucleotide sequence of the invention.

The recombinant sequence can be used both to clone a coding sequence in a given host, e.g. in a bacterium such as E. coli, and then to transfer this sequence in order to express it in a different host, e.g. an actinomycete, especially a strain of mycobacterium and in particular a BCG strain.

The invention can be used to clone or express any type of nucleic acid sequence, particularly sequences that code for peptides, polypeptides, or proteins (this set can be referred to by the term "polypeptide") having an antigenic character.

The invention relates, for example, to a particular recombinant sequence conforming to the above definition and characterized in that at least one nucleic acid sequence placed

under the control of a nucleotide sequence according to the invention (I, II, or III) as defined above, codes for a peptide or polypeptide that is immunogenic or capable of being rendered immunogenic.

By way of example, a nucleic acid sequence to be expressed which is incorporated in the recombinant sequence of the invention can be a sequence characteristic of a pathogenic organism. Pathogenic agents are, for example, viruses, parasites, and bacteria. The following can be mentioned in particular: M. leprae, M. tuberculosis, M. intracellulare, M. africanum, M. avium, the sporozoites and merozoites of plasmodium, the bacilli responsible for diphtheria, tetanus, Leishmania, Salmonella, certain treponemas, the toxin of pertussis and other pathogenic and viral microorganisms, particularly the mumps virus, rubella virus, herpes virus, influenza virus, the virus of Schistosoma, of Shigella, of Neisseria, of Borrelia, the rabies virus, the polio virus, the hepatitis virus, the AIDS virus (HIV, HTLV-I, HTLV-II, and SIV), and oncogenic viruses.

The nucleic acid sequence to be expressed can also code for an immunogenic sequence, for example, snake or insect venom.

Thus the recombinant nucleotide sequence can contain, under the control of the same nucleotide sequence of the invention, several antigen coding sequences, which can, if applicable, be characteristic of different organisms.

Preferably the invention relates to a recombinant sequence characterized in that the nucleic acid sequence to be expressed codes for a peptide or polypeptide of an HIV retrovirus, for example an envelope peptide or polypeptide, or a peptide or polypeptide of the Nef protein, of HIV-1 or HIV-2.

Other nucleic acid sequences can be used within the scope of the invention, and the following will be cited by way of example: the antigens or immunogenic sequences of mycobacteria, particularly the proteins or fragments of proteins

corresponding to genes involved in virulence and potentially protective antigens. An antigen is said to be "potentially protective" if it is capable of triggering or promoting a protective immune response, particularly by the production of antibodies or by the induction of a cell-mediated immune response, particularly of the TCL type.

One can envisage constituting a recombinant sequence in which the nucleotide sequence of the invention would involve itself in the control of the expression of one or more particular haptens or epitopes belonging, if applicable, to different organisms. Moreover, these haptens or epitopes can be associated with a sequence that codes for an antigen or in general for a polypeptide that can be used as a carrier protein, particularly for expression at the surface of a host cell, or indeed the secretion of the hapten(s) or epitope(s).

Association here means: the formation of a hybrid coding sequence from the different sequences present in the recombinant sequence or an association in the form of coding elements of an operon, with the different coding sequences retaining in this case their individuality during expression in a host cell or the formation of a protein fusion resulting from the expression of a gene fusion.

The nucleotide sequence I, II, or III of the invention can be placed either upstream of the nucleotide sequence to be expressed, and in phase with this sequence, or downstream of the nucleic acid sequence to be expressed. The choice of its position relative to the coding sequence can be determined as a function of the level of expression desired in a given host.

In the recombinant nucleotide sequence according to the invention, a nucleotide sequence according to the invention and the nucleic acid sequence or sequences to be expressed can thus constitute an operon fusion. In this case, if a number of nucleic acids are present, they are expressed under the control of the sequence but in the form of a single product.

According to another way of implementing the invention, the nucleotide sequence I, II, or III and the nucleic acid sequence or sequences to be expressed constitute a gene fusion. In this case the product of the expression of this gene is constituted by a hybrid protein or protein fusion when a number of nucleic acids are used.

The invention relates in general to the use of a nucleotide sequence according to the above description for the cloning and/or expression of nucleic acid sequences in a host cell other than an Mptb host cell, particularly in actinomycetes and notably mycobacteria, especially M. bovis; for example in the non-virulent BCG strain, gram-negative bacteria such as E. coli, and gram-positive bacteria such as B. subtilis.

Also included within the scope of the invention is a cloning and/or expression vector of the integrative type or replicative type, characterized in that it comprises — at a site that is non-essential for its integration or its replication — a nucleotide sequence comprising sequences I, II, or III or their variants, according to the above definitions.

These vectors are thus capable of replicating themselves extrachromosomally or, on the other hand, in the form of integrated elements within a chromosome or more generally within an element of the genome of a host in which they are incorporated, including in a plasmid or bacteriophage present in this host.

A vector according to the invention can also be characterized in that it is modified at a site that is not essential for its integration or replication, by an above-described recombinant nucleotide sequence.

A particular vector comprises, as well as the promoter and a heterologous sequence, at least one part of the sequence, designated ORF2, of Mptb (Figure 10). It is advantageous for this sequence to be placed downstream of the sequence containing the promoter, preferably between the promoter and

the heterologous nucleic sequence. Advantageously, this part of ORF2 corresponds to a sequence of 716 bp of Mptb as described in Figure 2.

By way of examples of satisfactory vectors for the implementation of the invention, the following can be mentioned: plasmids, transposons, phages, and any other vector that can be utilized for the expression of a sequence. In particular a plasmid that is of interest, if need be, as an intermediary plasmid for the implementation of the invention is an E. coli strain (Myc758) containing the fragment of 716 bp (PstI/BamHI) cloned in the vector pUC18, deposited in the "Collection Nationale des Microoganismes", Paris, France, under the number I-1157, on 23 October 1991.

This plasmid comprises, in particular, the promoter of the invention corresponding to sequence II, delimited by the restriction sites EcoRI/BglII (promoter pAN) and a linker sequence.

As examples of vectors, the following can be mentioned: the plasmids derived from pAL5000 (Ranzier et al, 1988, Gene 71: 315-321), RSF1010 (Hermans et al, 1991, Mol. Microbiol. 5: 1561-1566), pNG2 (Radford et al, 1991, Plasmid 25: 149-153), the transposons derived from Tn610 and IS6100 (Martin et al, 1990, Nature 345:739-743, IS900 (Green et al, Mol. Microbiol.), IS901 (Kunze et al, 1991, Mol. Microbiol. 5: 2265-2272), IS6110 (Thierry et al, Nucl. Acids Res. 1990, 18: p188), and the phages L5 (Lee et al, 1991, Proc. Natl. Acad. Sci., 88: 3111-3115) and D29 (Tokunaga et al, 1964, J. Exptl. Med., 119: 139-149).

Other elements of nucleotide sequences can be incorporated or present on the vector according to the invention, such as expression markers for example, and in this regard reference may be made to the genes of resistance to antibiotics such as kanamycin or viomycin. Other possible regulatory elements are: sequences involved in the expression of the coding sequence and in particular sequences of the operator type; or elements capable of promoting exportation, presentation at the level of

the host membrane, excretion, or secretion of the expression product of the heterologous sequence.

The invention also relates to a recombinant host cell characterized in that it is transformed by an above-described recombinant nucleotide sequence or by an above-described vector, in conditions enabling the expression of the nucleic acid sequence or sequences to be expressed which are contained in the recombinant sequence or in the vector.

A particularly advantageous host cell for the implementation of the invention is one which permits the presentation at its surface, or indeed the excretion or secretion, of the expression product of the nucleic acid sequence or sequences to be expressed that it contains or the synthesis of the expression product under conditions of intracytoplasmic localization.

Particular hosts are, for example: strains of actinomycetes, preferably non-virulent strains such as, for example, non-virulent BCG strain no. 1173P2 from the INSTITUT PASTEUR used to constitute the vaccine marketed under the name "Vaccin BCG Pasteur Mérieux".

The inventors have found, however, that the sequence containing the specific promoter from Mptb is capable of functioning in strains other than actinomycetes and, for example, can function in a gram-negative bacteria such as E. coli. This sequence is also able to be used in gram-positive bacteria such as B. subtilis or in Streptomyces.

A process suitable for the preparation of recombinant host cells according to the invention is, for example, electroporation according to the description by S. Snapper et al (1988, PNAS USA 85: 6985-6991) or conjugation according to e.g. the technique of R. Lazraq et al (1990, FEMS Microbiol. Lett. 69: 135-138).

Taking into account the advantageous properties of this promoter, and the possibility of expressing immunogenic sequences or antigens in selected strains and particularly in

non-virulent strains, the invention provides an immunogenic composition characterized in that it comprises a recombinant host cell meeting the above criteria, in a sufficient quantity to effect the production of antibodies or to contribute to the production of antibodies – preferably protective antibodies – in an animal or human host to which it is administered.

This immunogenic composition can be used to trigger a protective response against a particular pathogenic agent, by the production of antibodies, and the induction of a cell-mediated immune response when the expression product of the nucleic acid sequence expressed under the control of sequence I, II, or III is under conditions which enable the triggering of this production. The immunogenic composition can also be used as a booster composition to stimulate the production of antibodies initiated by a protein or other component.

The response engendered after the administration of the immunogenic composition can be of the cell-mediated type, particularly a TCL type sequence.

Thus the invention also enables the preparation of vaccines of the mixed-vaccine type, in which the production of antibodies will be directed both against the host cell, particularly the BCG bacillus, and against the expression product of the nucleic acid sequence to be expressed.

Besides its useful properties as a vaccine, a composition comprising a recombinant host cell according to the invention can be used for immunotherapy.

Such a vaccine or composition for immunotherapy can be used in an animal or in man and can be administered intradermally, subcutaneously, orally, in aerosol form, or percutaneously. Several administrations may be necessary, for example in booster form, to obtain sufficient protection.

A recombinant host cell according to the invention can also be used to produce any protein or peptide, particularly on an industrial scale. Thus the invention can be used for the production of antibiotics.

Other characteristics and advantages of the invention will become apparent from the examples and figures which follow.

FIGURES

Figure 1: Diagrammatic description of the construction of pAM320

The *Mptb* DNA is represented in grey by a double line and the lacZ gene is indicated by a double line.

pAM3 was digested with *Bam*HI/*Pst*I giving rise to a fragment of 716 bp which was recovered on 1% agarose gel using the Geneclean system. The fragment was ligated to the plasmid pNM482 digested by *Bam*Hi/*Pst*I, to produce pAM310. Competent strains of *E. coli* MC1061 were transformed with the ligation product, and the cells were spread on a Luria Broth (LB) medium containing 100 μg/ml of ampicillin. The clones bearing the recombinant plasmid pAM310 were recovered, checking the plasmid restriction chart. The fragment of 3.8 kb obtained from pAM310 by digestion with the enzymes *Sma*I/*Dra*I was eluted on 0.8% agarose gel and ligated by its free ends to the site *Sca*I of pRR3 to produce pAM320. In this construction the Ap^R gene (gene of resistance to ampicillin) was interrupted. The *E. coli* MC1061 cells were transformed and the colonies were selected by the Km^R Ap^S phenotype. The DNA of these recombinants was prepared by alkaline lysis and used to transform *M. smegmatis* mc^2155 (Snapper et al, 1990, Molec. Microbiol. 4: 1911-1919) by electroporation.

Figure 2: Nucleotide sequence of fragment BamHI/PstI of 716 bp obtained from pAM3 and the 185 N-terminal amino acids of ORF2

SD = Shine Dalgarno; +1 = initiation site of transcription.

Figure 3: ELISA on mouse sera taken 40 days after intravenous inoculation

Balb/C mice were immunized intravenously with 10^7 CFU of r-BCG transformed with pAM320, or with BCG strain 1137P2. A number of unimmunized mice were used as a control. The sera were taken 40 days after immunization and tested. The anti-β-galactosidase

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antibodies were detected by the ELISA method (E. Engval and P. Perlman, 1971, Immunochemistry 8: 871-874). The microtitre plates were covered with 1 μg of β-galactosidase per well. The anti-β-galactosidase antibodies were detected with goat anti-mouse immunoglobulin labelled with alkaline phosphate (Biosis). Each value corresponds to a pool of sera from four or five mice.

Figure 4: **Proliferative response of lymph node cells of a Balb/C mouse immunized subcutaneously with 10^7 CFU of BCG + pAM3 ■ , and the BCG 1173P2 strain ▨**

A group of immunized mice was inoculated with 0.1 ml of IFA. Two weeks later, the proliferative responses of LN cells (lymph node cells) to β-galactosidase, APH3', PPD, and ConA were tested.

Figure 5: **Proliferation of specific lymphocytes CD4+ and CD8+**

Balb/C mice were immunized subcutaneously with 10 CFU of BCG 1173 P2 (a) and r-BCG + pAM320 (b). Two weeks later, the proliferative responses of the LN cells to PPD (10 μg/ml) (a) and β-galactosidase (0.01 μg/ml) (b) were tested in the presence of monoclonal antibodies anti-CD4 (O) or anti-CD8 (O).

Figure 6: **Gamma-interferon responses of LN cells of mice**

a) mice immunized with BCG 1173P2

b) mice immunized with r-BCG (+ pAM320) after stimulation with 1 μg/ml β-gal. (o), 10 μg/ml PPD (o), 2.5 μg/ml conA (Δ).

Figure 7: **Cloning of promoter pAN adjacent to lacZ**

PCR on pAN
primers:
P1 = 5' CCCTCTAGAATTCCGTGACAAGGCCGAAGAGCCCGCGA 3'
P2 = 5' AACATATGAGATCTTCTCCTTCTGGGTTG[illegible]CCGCCCC 3'

2

PCR conditions, 35 cycles
Denaturing, 2 minutes at 95°C, pairing 2 minutes at 55°C, extension 2 minutes at 72°C.
Reaction volume 50 μl containing 10 μM of primers, 10 nmol of dNTP, and the target DNA of pAM3.

Figure 8: **Cloning of product of PCR in MCS of pSL 1180**

The product obtained by PCR was digested with XbaI/NdeI and ligated to pSL 1180 (Pharmacia) digested with XpaI/NdeI to give pWR30.

Figures 9a & 9b: **Diagrammatic description of the construction of pWR31, pWR32, and pWR33**

For Figures 7, 8, 9: PCR (gene amplification) was used to produce a fragment of 716 bp from pAM3 using primers P1 and P2. This fragment contained the promoter pam with sites XbaI and EcoRi at the 5' end and sites BglII and NdeI at the 3' end. The fragment was digested with XbaI and NdeI and cloned at the multiple cloning site (MCS) of pSL1180 to form the plasmid pWR30. At the time of preparation for the cloning of pAN at a site adjacent to the lacZ gene, the promoter pl and the CII gene were cloned preceding the truncated lacZ gene of pNM482. This enables the generation of a functional CII-LacZ fusion protein under the control of sequences upstream containing pL, and also a polylinker enabling subsequent constructions. The plasmid pTG952 was also digested with XHoI and its end was completed with the Klenow fragment. A fragment of 530 bp was then recovered from the plasmid after digestion with BamHI and cloned at the SmaI/BamHI site of pNM482. The resulting plasmid pIPJN thus possessed a functional gene of β-galactosidase under the control of the lambda pl promoter. In order to clone pAN in pIpJN, digestion by EcoRI/BGlII was performed on pWR30. The fragment of 159 bp was purified with Geneclean and cloned in pIpJN digested with EcoRI/BglII to give pWR31. When the *E. coli* strains were transformed with this plasmid and cultured in the presence of ampicillin and X-gal, blue colonies were formed. The operon fusion was then recovered from pWR31 by digestion with EcoRI/DraI. The EcoRI site was completed with the Klenow

enzyme and dNTP's, then ligated to the plasmid pRR3 digested by the enzyme ScaI. The two orientations of the operon in pRR3 were obtained. The transformation of E. coli and M. smegmatis with these two constructions (WR32, pWR33) enabled the formation of blue colonies when the bacteria were cultured in the presence of chromogenic substrate (X-gal).

Figure 10: Sequence of ORF2.

Figure 11: Response, in form of antibodies, on basis of serum of animals immunized with r-BGG expressing β-galactosidase.

Figure 12: Construction of plasmids containing pAN-ORF2-Nef(SIV) fusions. The plasmid pTG3148 is a vector derived from pTG959 (Guy et al, 1987, Nature 330: 266-269) in which a BglII fragment containing the nef gene has been cloned. This fragment was isolated from monkey cells containing the SIV virus (mac251) integrated as a provirus. The plasmid pBlue/pAM712 is derived from pBlueScriptIISK+ (Stratagene) in which the fragment BamHI/PstI containing pAN-ORF2 and excised from pAM1 has been cloned between sites BamHi and PstI of the polylinker.

Figure 13: Western-type analysis of the expression of the polypeptide ORF2-Nef(SIV). The molecular weight markers are indicated to the left of lane 1. Lane 1: Extract of baculovirus containing the protein Nef(SIV). Lane 2: BCG extract. Lanes 3 and 4: Two recombinant BCG extracts bearing the plasmid pSN25. Lanes 5 and 6: Two recombinant BCG extracts bearing the plasmid pSN26.

Figure 14: Analysis of proliferative responses of cells taken from lymph nodes of BALB/c mice inoculated with recombinant BCG bearing the plasmid pSN25. The immunization protocols are the same as those described in Winter et al, 1991 (Gene 109: 47-54). The degree of proliferation following in vitro stimulation with the different peptides is indicated on the vertical axis in Figure 3 according to their location on the protein (from N to C terminal). Three concentrations of peptides were used (0.1/1 and 10 μg/ml. The maximum proliferation was observed for one or other of these concentrations according to the peptides.

It is the maximum value which is indicated on Figure 3. Similarly, the proliferation was measured on the basis of mice immunized with non-recombinant BCG. The values obtained were always less than 2500 cpm (except for stimulation with peptide 84-96, for which a value of 5000 cpm was observed). The experiments carried out with unimmunized mice gave values lower than 250 cpm.

Figure 15: Plasmid pTG5167. This is a derivative of pT186 (Guy et al, 1987, op. cit.) in which a BglII-EcoRI fragment bearing the gene coding for the protein GP160 of HIV1/MN has been cloned in a polylinker.

Figure 16: Construction of plasmids pLA12 and pLA13 bearing the pAN-ORF2-Env(HIV1/MN) fusion.

Figure 17: Expression of polypeptide ORF2-Env(HIV/MN) by BCG bearing plasmids pLA12 and pLA13.

Figure 18: **Proliferative responses of the cells extracted from the lymph nodes of mice inoculated with BCG bearing the plasmids pLA12 and pLA13.**

Four Balb/c mice receive an intradermal injection of 10^7 CFU of BCG-pLA12, 4 others receive standard BCG 1173P2. After 14 days the peripheral lymph nodes are taken for the proliferation test.

Protein gp120 of HIV1-LAI is used to induce the proliferation of lymph-node cells which have been in contact with the protein fusion ORF2-region V3 produced by the recombinant BCG.

Figure 19: **Level of V3 antipeptide antibodies measured by ELISA tests**

Five mice receive an intravenous injection of a vaccine preparation containing 5 x 10^6 CFU of BCG transformed with pLA12; 5 others receive the same quantity of recombinant BCG pLA13; 5 mice receive a similar quantity of BCG recombinants expressing the lacZ gene but not the ORF2-env' gene; "naive"

mice receive no injection. Blood samples are taken after 14 days, then every 2 weeks, in order to measure the trend of the level of specific antibodies of the protein fusion ORF2-env'.

In the ELISA test we used a peptide of 20 amino acids of the V3 loop of $gp120_{MN}$ (aa306 to aa325), recognized by the antibody SC-D (K24-1), to serve as an antigen capable of being recognized by the antibodies present in the sera.

Figure 20: Plasmid pTG2103. This is a derivative of pTG959 (Guy et al, 1987, op. cit.) in which a fragment BglII/EcoRI bearing the gene coding for protein P24 of HIV1/LAI has been cloned in the polylinker.

Figure 21: Construction of the plasmids pLA22 and pLA23 bearing the fusion pAN-ORF2-gag(HIV1/LAI).

Figure 22: Expression of the polypeptide ORF2-Gag(HIV1/LAI) by BCG.

MATERIAL AND METHODS

Bacteria-strains, phages, plasmids, and culture media

M. bovis BCG strain Pasteur 1173 P2, known as BCG (A. Calmette, "L'infection bacillaire et la tuberculose chez l'homme et chez les animaux. Processus d'infection et de défence. Etude biologique et expérimentale. Vaccination préventive." Ed. Masson. Paris 1928) was used as a host for the construction of different BCG recombinants (r-BCG). Other bacteria, phages, and plasmids used in the experiments reported below are described in Table I. The BCG was cultured till the formation of plaques on Sauton medium (Sauton, B., Comptes Rendus Académie des Sciences 1912: 155-1860 [sic] in Calmette, A., et al, "Vaccination préventive par la BCG " p. 811, Ed. Masson 1928) to produce a classic vaccinating preparation or in the form of a disperse culture. The bacterial clones of r-BCG were first cultured on Lowenstein-Jensen medium (K. Jensen, Towards a Standard of Laboratory Methods, 4th Rep. Subcomm. Laboratory Methods. Bull Union Internat. against Tuberc., 1957,27: 146-166) containing 10 µg/ml of kanamycin, then transferred to Sauton medium. These cultures were used for *in vitro* analyses of expression and for immunization of the animals. The *M. smegmatis* and *E. coli* strains were cultured according to the method described by Ranes et al (1990) J. Bact. 172: 2793-2797.

Preparation of DNA of M. paratuberculosis

50 mg of lyophilized *M. paratuberculosis* strains (A. Murray et al, NZ Vet Journal op. cit.) were resuspended in an homogenization buffer solution (0.1M NaCl, 0.03M Tris-HCCL, pH 7.5, 0.006M EDTA), mixed with 2 ml of glass balls (diameter 0.45-0.5 mm) and agitated in a Braun rotary homogenizer at maximum speed for 2 minutes at 4°C. After centrifugation and extraction with phenol/chloroform, the DNA was precipitated with 95% ethanol and treated with 0.1 mg/ml of DNase without RNase. After a further extraction with phenol/chloroform, and

precipitation in ethanol, the DNA was resuspended in water (A260/280 ratio = 1.8).

Construction of gene library of *M. paratuberculosis* genes

The method used was based on the protocols described by Young et al (Proc. Natl. Acad. Sci. (1985) 82: 2583-2587) modified by Murray et al (NZ Vet. J. (1989) 37: 47-50). The gene library contained 2.2 x 10^5 recombinant bacteriophages. After amplification in *E. coli* Y1090, the recombinants represented approximately 85% and corresponded to a titer of 3 x 10^{11} PFU/ml.

Screening of the lambda gt11 library of *M. paratuberculosis*

The library was screened by differential hybridization of the DNA obtained by transfer to membrane from Petri dishes containing lysis phages (Young et al, cited above), using the complete chromosomal DNA of *M. paratuberculosis* (*Mptb*) and *M. phlei* for probes. The recombinant bacteriophage, which gave a positive signal after hybridization with the DNA of *Mptb* but did not hybridize with *M. phlei*, was kept for subsequent analyses. It was propagated using the lysate-on-plate method as described by Maniatis et al (J. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory. Cold Spring Harbor. N.Y.(1982)).

Manipulation of the DNA

The DNA fragments were separated on 0.8% or 1% agarose gels containing 0.5% ethidium bromide. The DNA was eluted from the gels and purified using the Geneclean II kit (Bio101 Inc, La Jolla) according to the manufacturer's instructions.

The cloning of the fragments in plasmids and the transformation of *E. coli* were performed using the standard techniques of Maniatis et al (publication cited above). The transformation of mycobacteria was performed according to the description of Ranes et al (cited above).

Determination of the nucleotide sequence

The DNA was cut at random by sonication and the fragments were cloned blind in M13. The clones were then sequenced by the chain-termination dideoxy method using the Sequenase kit (USB) and 7-deaza-dGTP, an analogue of dGTP. Overlaps and data analysis on the sequences on the basis of the fragments obtained at random were performed using the computer programs described by Staden et al (Nucleic Acids Res. (1986) 14: 217-231).

Preparation of RNA

A culture of 100 ml of *M. smegmatis* MYC760 strains was cultured up to the mid-log phase in a 7H9 medium, completed with Middlebrook ADC enrichment (Difco) plus 25 μg/ml of kanamycin at 37°C. The cells were collected after centrifugation, washed with the same fresh growth medium, and resuspended in a medium containing 1% (w/v) sodium triisopropylnaphthalene sulfonate and 6% (w/v) sodium 4-amino salycilate. 14 g of glass balls (4.5-5.5 mm) were added, and the mixture was agitated vigorously in a vortex apparatus for 2 x 2 minutes. The supernatant was extracted two times with phenol/chloroform and the nucleic acids within the aqueous phase were precipitated with propan-2-ol in the presence of 0.3 M sodium acetate at pH 6. After centrifugation for 10 minutes at 8000 rpm, the centrifugation pellet was washed with 100% ethanol, dried at ambient temperature for 10 minutes, and resuspended in 1 ml of distilled water treated with DEPC. The RNA was precipitated in the presence of three volumes of 4 M sodium acetate (pH 6) at -20°C for 18 hours, collected by centrifugation and resuspended in distilled water (optical density OD 260/280 2.0). The RNA was preserved at -20°C in the form of a precipitate in propan-2-ol.

Mapping of the transcripts

The plasmid pAM311 was linearized with BssHII to produce 5′ extensions, and dephosphorylated with calf-intestine alkaline phosphatase. After purification of the plasmid with

the Geneclean kit, the DNA was cut with the restriction enzyme PstI and the fragment of 3.1 kb was isolated from the 1% agarose gel. The hydroxyl 5′ end was radio-labelled with ATP (gamma^{32}P) (specific activity 3000 Ci/nmol) using the polynucleotide kinase (10 units). The unincorporated tracer was removed by passage through a Nick column (Pharmacia).

The RNA (40 µg) and the radio-labelled DNA probe (0.1 µg) were mixed in a total volume of 30 µl of distilled water, 240 µl of deionized formamide were added, and the mixture was heated to 100°C for 3 minutes. After rapid cooling on ice, 30 µl of hybridization buffer solution, 10 x (0.2 M PIPES-NaOH, pH 6.4, 4M NaCl, 20 mM EDTA), were added and incubation was continued at 60°C for 3 hours. The DNA/RNA hybrids were precipitated with three volumes of ethanol at -20°C for 16 hours. After centrifugation for 15 minutes at 4°C, the pellet of nucleic acid was resuspended in 100 µl of buffer solution containing 50 mM of sodium acetate at pH 4.6, 280 mM of NaCl, 5 mM of $ZnCl_2$, and 20 µg per ml of denatured salmon-sperm DNA. 2 µl of nuclease S1 (472 units) were then added and digestion was continued for 30 minutes at 20°C. The reaction was arrested by the addition of 25 µl of a solution containing 2.5 M CH_3COONH_4 and 50 mM EDTA at pH 8. The DNA/RNA hybrids precipitated with propan-2-ol in the presence of 1 µg of carrier DNA (denatured salmon-sperm DNA). After washing in 80% ethanol, the pellet was resuspended in 5 µl of distilled water and 7 µl of stop solution (95% v/v formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF). The mixture was heated to 100°C for 5 minutes and then put on a sequencing gel with 6% polyacrylamide.

Serological tests

Murine sera were tested by ELISA to detect specific antibodies directed against β-galactosidase according to the following technique: 96-well microtiter plates (Nunc) were covered with 10 µg/ml of β-galactosidase purified in PBS buffer solution for 1 hour at 37°C and for 16 hours at 4°C. After washing three times with PBS containing 0.1% Tween 20, the sera preabsorbed with the BCG extracts for 16 hours at 4°C were

added to the wells in a dilution buffer (PBS + 0.1% Tween 20, 1% BSA) for 2 hours at 37°C. After three washings, the antibody titers were determined by photometry at 405 nm using a rabbit anti-mouse IgG alkaline phosphatase conjugate (Biosys, Compiègne) and 1 mg/ml of p-nitrophenyl phosphate as substrate.

β-galactosidase test

The β-galactosidase activity was measured in *E. coli* cells treated with toluene and in extracts of *M. smegmatis* subjected to treatment with ultrasound according to the description by Cossart et al (J. Bacteriol. (1985) 161: 454-457.

Immunization of animals

Six-week-old female Balb/c mice were obtained from Iffa Credo. In order to track the cell-mediated immune responses, the mice were inoculated subcutaneously at the base of the tail with 10^7 colony-forming units of BCG strains. Specimens of lymph node cells were taken 14 days after immunization and the proliferative responses were studied. A control group of mice received incomplete Freund adjuvant (IFA) in a saline solution. To track the production of antibodies, a group of mice was inoculated intravenously with 5 x 10^6 CFU of BCG strains. Certain mice received an intravenous inoculation of booster three times at 21 day intervals with 10^6 CFU; the serum samples were taken 28 days after immunization and 14 days after each booster to titrate the antibodies.

The stability of the different strains of BCG was analysed by determining the number of BCG colony forming units recovered from the spleen two months after intravenous inoculation with 10^7 CFU of BCG.

Proliferative responses to specific antigens

Fourteen days after immunization, the cell suspensions were prepared from inguinal lymph nodes taken from three mice and resuspended in RPMI1690 (Gibco) containing 2 mM L-glutamine, 50 μg/ml gentamicin, 5 x 10^5 M 2-mercaptoethanol, and 10% fetal calf serum (FCS). The lymph node cells were

cultured to a concentration of 4×10^5 cells per well in flat-bottomed culture trays containing 96 wells (Corning) in the presence of an appropriate antigen. The concentration of the antigens used was as follows: 0.01 µg/ml of APH3′ and β-galactosidase and 10 µg/ml of a purified protein derivative (PPD). Concanavalin A (ConA) was added at a concentration of 2.5 µg/ml, to serve as a non-specific positive reaction control. Certain cell suspensions remained unstimulated. Each test was performed in triplicate. The cultures were incubated for five days at 37°C, the last 22 hours being in the presence of tritiated thymidine methyl (3H dThd 1mCi = 37KB2 Amersham), 0.4 uci/well, in an atmosphere of humidified air containing 7% CO_2. The cells were then collected on glass-fibre filters (Automash 2000 Dynatch) and the incorporated radioactivity was measured. The results were expressed in counts per minute (cpm) with the background noise subtracted. The standard errors of the mean of the cultures in triplicate were determined. The background noise values for the non-stimulated control cultures were less than 10^4 cpm.

Anti-CD4 and anti-CD8 monoclonal antibodies

In order to determine the ratio of the subgroup of T cells involved in the proliferation, monoclonal antibodies against the subgroups of T cells were added to the cultures of lymph node cells at different concentrations. L3T4 (CD4+) (specific rat anti-mouse CD4+ hybdridoma GK 1-5) and LYT2 (CD8+) (specific rat anti-mouse CD8+ hybdridoma H35 17-2) were produced according to the method described by D.P. Dialynas et al, 1984, J. of Immunology, Vol 31, p. 2445-2451). Briefly, to obtain ascites of monoclonal antibodies, nude mice were inoculated once with cells corresponding to 106 hybridomas. The antibodies were collected by precipitation in ammonium sulfate. The quantity of proteins was determined by optical density at 280 nm.

Measurement of cytokines

The synthesis of gamma-interferon was measured in the supernatant of the cultures of lymph node cells at the

conclusion of the proliferation test. The level of gamma-interferon was determined by a solid-phase immunoenzyme test, using the multiple sandwich principle (Genzyme). The supernatant was diluted (1/2-1/10). The standard gamma-interferon was diluted to obtain values inside the linear interval of the test (128-8200 pg/ml).

RESULTS

Isolation and characterization of recombinant bacteriophages

A lambda gt11 genomic library was constructed to isolate pecific sequences of M. paratuberculosis (Mptb). A recombinant hage which hybridized strongly with chromosomal DNA of Mptb ut not with DNA from M. phlei was taken individually and used o prepare stocks of lysates of phages. (Maniatis et al, cited bove). One of these recombinants was selected at random for dditional tests. Its genome contains an Mptb insertion of 3.8 b. The mycobacterial DNA was recovered from this fragment by igestion with the restriction enzymes EcoRI and BamHI. This ed to the obtaining of four fragments, which were separated on garose gel. One of these fragments, of 1.6 kb, was eluted from he gel and ligated to the plasmid pGEM-2 digested by coRI/BamHI. Competent cells of E. coli DH5 alpha (Bethesda esearch Laboratories, Gaithersburg, MD, USA) were transformed ith the ligation mixture according to the manufacturer's nstructions and then applied to trays with an LB medium ontaining 100µg/ml of ampicillin. A single colony was selected nd used to prepare a sufficient quantity of recombinant lasmid, which was designated pAM3, using the standard echnique described by Maniatis et al, cited above.

When pAM3 was labelled with the chemical probe kit (Promega) and used as a probe in the dot-blot test, hybridization occurred solely with the DNA extracted from M. paratuberculosis. The different Mptb isolates tested included the M. paratuberculosis type strain, isolates of 23 Mptb's of bovine, ovine, and caprine origin, and an isolate from a human patient with Crohn's disease. The pAM3 plasmid did not hybridize with M. avium serovar 2 and 3, M. intracellulare, M. tuberculosis strain H37Rv, M. bovis, M. phlei, M. smegmatis, or N. asteroïdes.

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Sequence analysis

The nucleotide sequence of the BamHI/PstI fragment of 716 bp is reported in Figure 2. Analysis of this sequence to look for the consensus signals for the start of transcription and translation revealed a potential region at -35 (position 83-88), a region at -10 (position 106-111), and a Shine Dalgarno (SD) sequence at position 147-152. A long open reading frame (ORF) designated ORF2 follows the SD sequence with an initiation codon ATG at position 160. Analysis of the nucleotide sequence of the fragment revealed homology with the *M. paratuberculosis* IS900 insertion element. Segment 153-716 was identical to sequence nt1451 to 888 of IS900 (Green et al, (1989) Nucl. Acids. Res. 17: 9063-9073). ORF2 is in the opposite direction to the transcription of the potential transposase of this element, ORF1197. Segment 1-152 is outside the sequence of IS900.

ORF2 presents no similarity with other sequences, if we refer to the sequences contained in the database. When IS900 is used as a genomic probe for Southern blot tests of different Mptb isolates, almost identical multiple band diagrams are obtained, irrespective of the origin of the isolates (McFadden et al (1987), J. Clin. Microbiol. 25: 796-801). This absence of polymorphism suggests that IS900 is integrated in a predominant manner at specific sites inside the Mptb genome. A limited number of sequences adjacent to the termination of IS900 have been reported, and seem to contain conserved nucleotide sequences (Green et al (1989), Nucl. Acids Res. 17: 9063-9073). The Shine Dalgarno sequence AAGGAG shown in Figure 2 is adjacent to the IS element. The inverse sequence complementary to this SD sequence (CTCCTT) is identical to the flanking sequence reported for IS900 contained in the clone pMB22 derived from the genomic library of the isolate from a human patient with Crohn's disease.

Analysis and expression of ORF2

To study the expression of ORF2 at a transcription and translation level, the BamHI/PstI fragment of 716 bp of pAM3 was cloned in between the BamHI and PstI sites of the fragment containing multiple cloning sites (MCS) of the plasmid pNM482 (Minton et al (1984), Gene 31: 269-273). The MCS is adjacent to a truncated β-galactosidase (lacZ) gene without its first 8 terminal amino acids and its transcription and translation starting signals. The introduction of a correctly aligned segment containing appropriate regulatory signals and a coding sequence downstream, enables the formation of a hybrid gene that confers the LacZ$^+$ phenotype. ORF2 was in phase relative to the lacZ gene whose construction is described above and the translation product should have been able to be expressed in the form of an ORF2-β-galactosidase protein fusion containing 185 ORF2 amino acids. However, the transformation of E. coli MC1061 did not produce colonies transformed by Lac$^+$. The expression of the ORF2-lacZ gene fusion in a mycobacterial system was studied. Consequently the SmaI/DraI fragment of 3.8 kb containing the gene fusion was ligated in the form of a fragment with a free end, at the ScaI site of the plasmid pRR3, which is a shuttle plasmid between the mycobacteria and E. coli for the production of pAM320. The transformation of M. smegmatis with this plasmid by electroporation, followed by culture in the presence of kanamycin and X-gal, led to the obtaining of blue colonies, which appeared in three days. A similar experiment to transform BCG with this plasmid also resulted in blue colonies, which appeared in approximately 21 days. Thus ORF2 was able to be expressed in the form of a protein fusion with β-galactosidase.

To quantify the expression of ORF2, the level of activity of β-galactosidase was measured in M. smegmatis and E. coli transformed with pAM320 or pRR3. The results are given in table III. 250 units were found in M. smegmatis transformed with pAM320 harbouring the ORF2-lacZ gene fusion but no activity was found in the M. smegmatis strain transformed with pRR3. In the E. coli strains transformed with pAM320, only a low level of

activity was found (5/7 units). These results show that the ORF2-lacZ fusion is not expressed in E. coli and suggests that ORF2 must be specifically expressed in the mycobacteria.

Analysis of transcription

The start site of the transcription of the ORF2-lacZ gene fusion inside pAM320 was determined by high-resolution mapping with the nuclease S1. The experiments showed that the start site of the transcription of the sequence is situated upstream of ORF2 at position 119 (Figure 4). A second starting site is present outside the microbial sequence. Two explanations can be found for this observation. Firstly, it could be due to hybridization of the probe with a small quantity of shuttle plasmid co-precipitated during the preparation of the RNA; otherwise, it could be due to reading the results of transcription per medium of the ampicillin resistance gene of pRR3. These results, and also the expression of the protein fusion with β-galactosidase, indicate that the promoter element pAN which is adjacent to the element IS900 is capable of controlling the expression of ORF2. This is the first demonstration of the induction of the expression of a gene located inside an insertion sequence, by means of an adjacent chromosomal promoter.

Construction of the ORF2-lacZ operon fusion

To characterize the activity of the promoter pAN more precisely, ORF2 was deleted and an operon fusion with the lacZ gene was constructed (Figures 7, 8, 9).

pWR32 and pWR33 are recombinants of pRR3 containing the pAN-LacZ fusion in both orientations (Figure 9). The transformation of either E. coli or M. smegmatis with these constructions, followed by culture in the presence of kanamycin and X-gal, led to the obtaining of blue colonies for both species of bacteria. Thus pAN is functional in E. coli when it is present in an operon fusion with Lac-Z but it is not functional when it is present in a gene fusion with ORF2.

pWR32 induced the same level of β-galactosidase activity in M. smegmatis as pAM320. However, the level of activity with pW33 was 10 times stronger in E. coli. This must be due to the constitutive action of a promoter upstream of the pAM-lacZ gene fusion.

Specific cell-mediated immune response

Balb/c mice were inoculated subcutaneously with BCG recombinants harbouring the pAM320 plasmid expressing the phosphotransferase APH3' under the control of its own regulatory region and lacZ under the control of pAN. The proliferative responses of the lymph node cells taken 14 days after immunization were analyzed. A specific response to an in vitro stimulation with different antigens was observed (Figure 4). Only the lymph node cells of mice immunized with r-BCG expressing lacZ and APH3' proliferated in response to in vitro stimulation with β-galactosidase and aminoglycoside phosphotransferase (APH3'). The cell proliferation in response to an extract of PPD (Protein Purified Derivative) was similar with the lymph nodes of the mice immunized with the non-recombinant BCG strain. The lymph node cells of the unimmunized animals only proliferated in response to ConA. This non-specific proliferation was of the same order of amplitude in all the groups of animals.

It was shown that the T cells CD4+ and CD8+ are involved in the proliferative responses described above, by alternating inhibition of proliferation, with monoclonal antibodies anti-CD4+ and anti-CD8+. The results appear in Figure 5. Inhibition of 70% of the specific response to β-galactosidase is observed after the addition of anti-CD4 antibodies to the cultures of lymph node cells. In similar experiments, 30% inhibition was observed after addition of anti-CD8 antibodies to the cultures. These results show that the strongest response is obtained by the subgroup of CD4+ T cells. Two different populations of CD4+ T cells participate in the regulation of the immune response in mice. A subgroup of CD4+ T cells designated TH1 produces interleukin-2 (IL2) and gamma-interferon and preferentially activates the macrophages to kill or inhibit the intracellular

growth of the pathogenic agent. Another subgroup of CD4+ T cells designated TH2 produces other lymphokines, including IL4, IL5, and is involved in the induction of humoral responses. Significant production of gamma-interferon was detected in the supernatant of cultures of lymph node cells stimulated with specific antigens (Figure 6). These values were slightly lower than those obtained after stimulation with PPD or ConA. However, they remain significant, as the lower limit of the standard curves was 100 pg/ml. The production of gamma-interferon by the lymph node cells isolated from the animals immunized with non-recombinant BCG was only observed after stimulation *in vitro* with PPD or ConA.

Antibody response

Blood was taken four weeks after intravenous inoculation with the two layers of BCG and 14 days after each of the intravenous boosters. The antibodies against β-galactosidase were detected by an ELISA test on sera from animals immunized with r-BCG, expressing β-galactosidase (Figure 3). An increase in the antibody response was observed after the different boosters. The results appear in Figure 11. A major increase in the level of antibody is observed after the first and then the second booster. A third booster does not provoke any increase in the level of antibody. The antibodies against β-galactosidase are detected in the sera of animals immunized with non-recombinant BCG. This response is much weaker than the response induced by r-BCG expressing β-galactosidase and may be due to polyclonal activation by the BCG. No antibody was detected in non-immunized mice. These results demonstrate that the humoral response can be triggered by r-BCG expressing a foreign antigen (heterolog) under the control of pAN. Beta-galactosidase was chosen as a model system but it can be replaced with any type of antigen that is of interest for the purposes of vaccination.

Stability in vivo of the different r-BCG strains

The BCG bacilla were recovered from spleen homogenates two months after intravenous inoculation. Table II shows that the

different r-BCG clones used in this study have a similar behaviour to that of the non-recombinant BCG strain. After spreading the r-BCG strains on media containing kanamycin and X-gal, 2.0 x 10^5 blue CFU's were obtained compared with 7.4 x 10^5 CFU's after culturing in a medium without selection by kanamycin. Thus about 27% of the r-BCG population is stable after two months growth <u>in vivo</u>. This proportion must enable the multiplication and persistence of BCG in the macrophages of the target organ, these phenomena being required for long-term immunogenic stimulation.

<u>Conclusion</u>

The results presented here demonstrate that the strains of r-BCG harbouring plasmids that code for APH3' under its own control region and β-galactosidase under the control of pAN can trigger cell-mediated and humoral immune responses, specific for these antigens, in mice. These polypeptide antigens are located in the cytoplasm of r-BCG. As has already been described for BCG, the derivatives of r-BCG must multiply inside the macrophages and present the peptides of β-galactosidase in association with the molecules of CMH. This leads to recognition by the T lymphocytes, which respond by proliferating. The lymph node cells showed strong proliferation in response to stimulation <u>in vitro</u>. The T cells CD4+ and CD8+ proved to be involved in the proliferative response with a percentage of 70% CD4+ cells and 30% CD8+ cells. The production of gamma-interferon suggests that an efficacious role is played by the TH1 cells subgroup. These cells are responsible for the required activation of the macrophages to eliminate the intracellular pathogenic agents. The antibody titers found also suggest the co-operation of the subgroup of T cells designated TH2, which plays a part in the induction of the humoral responses.

The fact that 27% of these r-BCG strains are recovered without major rearrangement, after two months of growth <u>in vivo</u> in mice, suggests that they will permit the induction of a memory-based immune response. The subsequent cloning of the antigens on the chromosome, using different methodologies such

as transposition, homologous recombination, and integration mediated by a phage or plasmid, will enable the construction of r-BCG strains having an even more satisfactory stability and inducing more persistent long-term immune responses due to continuous stimulation.

Table I

BACTERIAL STRAINS, PHAGES, AND PLASMIDS

Bacteria	Description	Source or Reference
M. paratuberculosis	Bacterial strain isolated from a bovine with Johne's disease	Massey University, New Zealand
M. smegmatis mc^{2}155	Mutant of mc^{6} with high transformation-efficacity	Snapper et al.
M. bovis BCG	Pasteur BCG 1173P2 strain	Institut Pasteur, Paris
E. coli Y1090	Receptor strain for lambda gt11	Marketed by Promega
E. coli MC1061	Receptor strain for transformation by plasmids replicating in *E. coli*	Maniatis et al
E. coli DH5α	Receptor strain for transformation by plasmids replicating in *E. coli*	Maniatis et al
Phage		
Mptb lambda gt11	Genomic DNA library of *Mptb*	Murray et al
pUC18	Vector with large number of copies	Murray et al
pGEM-2	Plasmidic vector with large number of copies	Marketed by Promega
pNM482	Vector for detection of promoter	Minton et al
pRR3	Shuttle vector, *E. coli*-mycobacteria	Ranes et al
pAM-3	Recombinant pGEM-2 containing a fragment *Eco*RI/*Bam*HI of 1.6 kb of *Mptb*	Described in text

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Table I (cont.)

BACTERIAL STRAINS, PHAGES, AND PLASMIDS

Bacteria	Description	Source or Reference
pAM310	Recombinant pNM482 containing a fragment BamHI/PstI of 716 bp of pAM-3	Described in text
pAM-320	Recombinant pRR3 containing a fragment DraRI/SmaI of 3.8 kb of pAM310	Described in text
pAM-311	Recombinant pUC_{18} containing a fragment BamHI/PstI of 716 bp of pAM-3	Described in text
pAM-312	Recombinant pUC18 containing a fragment EcoRI/BamHI of 1.6 kb of Mptb	Described in text
pSL 1180	Derivative of pUC18	Pharmacia
pWR30	Recombinant pSL 1180 containing product of digestion by PCR of XbaI/NdeI (168 bp)	Described in the text
pTGT 959	Plasmid containing the promoter of lambda PL	Transgene
pIpJNI	Recombinant pNM482 containing the XhoI/BamHI fragment of pTG 959	Described in the text
pWR31	Recombinant pIpJN containing the EcoRI/BLGII fragment of pWR30 (159 bp)	Described in the text
pWR32 + pWR33	pRR3 recombinants containing the EcoRI/DraI fragment (3.8 kb) of pWR31 in both orientations	Described in the text

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Table II

In vivo stability of r-BCG (+ pAM320)

BCG cfu's recovered from homogenates of mouse bone marrow, 2 months after intravenous inoculation with 10^7 CFU kanamycin

r-BGC (+pAM320) Clone	Culture medium 7H11	Culture medium 7H11 + Kan + X-gal
39.3	759000	199000
39.4	720000	194000
BCG 1137P2	740000	0

Table III

Beta-galactosidase activity (units/mg*)

Recombinant host organism

Plasmid	*M. smegmatis*	*E. coli*
pRR3	0	not given
pAM320	250	5-7
pWR32	250	350
pWR33	350	2500

* Dry weight, determined by the optical density at 600 nm (1 mg, dry weight, per ml = 3.7 optical density units at 600 nm)

Examples of use of the pAN promoter, associated with ORF2: expression of viral antigens and induction of specific immune responses by BCG expressing these antigens.

The promoter pAN associated with the open reading frame ORF2 was used to express a number of viral antigens in BCG. Immune responses against these viral antigens were observed after inoculation of mice with strains of BCG expressing them.

1) The nef gene of SIV

The entire nef gene of SIV was inserted at the PstI site of ORF2. To do this, a fragment containing the nef gene of SIV was synthesized in vitro by PCR. Two oligonucleotides SN1

(5'CCCCTGCAGAGATCTATGGGTGGAGCTATT3') and SN2

(5'AAAAAGCTTTTAGCCTTCTTCTAACTT3')

(G. Myers et al [editors], "Human Retroviruses and AIDS, 1991, A Compilation and Analysis of Nucleic Acid and Amino Acid Sequences", Theoretical Biology and Biophysics Group T-10, Los Alamos National Laboratory, Los Alamos, New Mexico, USA) were synthesized by the APPLIGENE company. They served to amplify the nef gene of SIV from the plasmid pTG3148 bearing it which came from TRANSGENE (Figure 12). The amplified fragment bears the restriction sites PstI and HindIII. It is cut with the corresponding restriction enzymes and cloned in the plasmid b/pAM712 (Figure 12). The recombinant plasmid bearing nef of SIV was designated pSN24. From this plasmid, the XbaI/HindIII fragment bearing the nef gene of SIV was cut with the enzymes XbaI and HindIII, the ends were filled with the Klenow enzymes, and the resulting fragment was cloned at the site ScaI of pRR3, in both orientations. The resultant plasmids were called pSN25 and pSN26.

Plasmids pSN25 and pSN26 were then transferred to M. smegmatis and the BCG by electroporation. The polypeptide fusion ORF2-Nef is located by western blot using sera from monkeys infected with SIV (Figure 13). After inoculation of mice with BCG expressing ORF2-Nef(SIV), a cell-mediated immune

response against Nef(SIV) was observed using lymph node cell proliferation tests after stimulation by Nef(SIV) peptides. The peptides come from ANRS. These are the peptides 1-15; 16-30; 40-60; and 221-235 (the numbers correspond to the location of the N-terminal and C-terminal amino acid on the entire nef protein). The range of responses obtained with the different peptides is indicated in Figure 14. It is probable that it is a question here of T(CD4) and T(CD8) lymphocytes as we showed previously for Nef of HIV1.

2) The env gene of HIV1, MN strain

To do this, the cloning was performed similarly to the cloning of nef of SIV. The gene fragment coding for the polypeptide of amino acid 242 to 335 of the protein GP120 was synthesized in vitro by PCR.

Two oligonucleotides:
JENVMN3:5'CGACTGTAAAAATGTACTGACGTCCCCC3' and
JENVMN4:5'TAAAAGCTTTTACTCGGTGTCGTTCGTGTC3'
(G. Myers et al [editors], "Human Retroviruses and AIDS, 1991, A Compilation and Analysis of Nucleic Acid and Amino Acid Sequences", Theoretical Biology and Biophysics Group T-10, Los Alamos National Laboratory, Los Alamos, New Mexico, USA) were synthesized by APPLIGENE. They serve to amplify the env gene from the pTG5167 plasmid constructed by Transgène (Figure 15). The amplified fragment bears the restriction sites PstI and HindIII. It is cut with the corresponding restriction enzymes and cloned in the plasmid b/pAM712 between sites PstI and HindIII (Figure 16). The resulting plasmid was called pLA11. It contains gene fusion ORF2-env containing the 554 bp corresponding to the N-terminal part of ORF2 and the 686 bp corresponding to the N-terminal part of the env gene. The pAN-ORF2-env fusion was excised from pLA11 by a double cut with the enzymes BamHI and HindIII. The ends of the fragment were filled with the Klenow polymerase. The resulting fragment was cloned in pRR3 at the ScaI site, giving rise to the plasmids pLA12 and pLA13 according to the orientation of the insert. The plasmids pLA12 and pLA13 were introduced by electroporation into M. smegmatis and BCG. The expression of the ORF2-Env fusion was

detected by western blot using the monoclonal antibody SC-D(K24-1) from HYBRIDOLAB. As shown in Figure 17, an expression of polypeptide fusion of the expected molecular weight (45.5 kDa) was observed.

Mice were inoculated with the BCG expressing the ORF2-Env fusion. A fortnight after inoculation, an in vitro proliferative response of the lymph node cells was observed after stimulation with the protein gp120 (Figure 18). Probably it is here again a question of T (CD4) and T (CD8) lymphocytes. Other mice were inoculated intravenously, and a booster was given at the end of 28 days. Blood was taken at different times after injection. A fortnight after the booster, an elevated level of antibody was detected by the ELISA test, using the protein g120 or peptides corresponding to the part of Env expressed by the BCG (Figure 19).

3) The gag gene of HIV1, LAI strain

Using constructions similar to those set out above, the part of the gag gene that codes for the protein P24 (the first 217 amino acids) of the HIV LAI virus was inserted at the XhoI site inside ORF2. To do this, a fragment containing the gag gene was synthesized in vitro by PCR using the EML3 oligonucleotides:

5'GGGCGCGCTCTCGAGTATGAGAACTTTAAATGCA3' and

EML5:5'GTTCGAATTCTCACAAAACTCTTGC3'

(G. Myers et al [editors], "Human Retroviruses and AIDS, 1991, A Compilation and Analysis of Nucleic Acid and Amino Acid Sequences", Theoretical Biology and Biophysics Group T-10, Los Alamos National Laboratory, Los Alamos, New Mexico, USA) and the plasmid pTG2103 constructed by Transgène (Figure 20) as a matrix. This fragment containing the gag gene was cut with the enzymes XhoI and EcoRI and cloned between the XhoI and EcoRI sites of the plasmid pAM1 (Murray et al, 1992), thus achieving an ORF2-Gag fusion, and giving rise to the plasmid pLA21 (Figure 21). From this plasmid, the BamHI/EcoRI fragment bearing the ORF2-Gag fusion was excised from pLA21 by cutting with the enzymes EcoRI and BamHI. The ends of the fragment were then filled with the Klenow polymerase and the resulting

fragment was cloned in pRR3 at the ScaI site to give rise to the plasmids pLA22 and pLA23. These plasmids were transferred by electroporation to *M. smegmatis* and BCG. The ORF2-Gag fusion was expressed in the form of a polypeptide of 46.5 kDa by *M. smegmatis* and the BCG (Figure 22).

WHAT WE CLAIM IS:

1. An isolated nucleotide sequence which in use enables the expression, in a host cell, of a given nucleotide sequence under its control, characterized in that it comprises a sequence selected from:

   (a) the following sequence or any part thereof:

```
GAT CCC GTG ACA AGG CCG AAG AGC CCG CGA CCG TGC GGT CGT CGA CGA

                                                (-35)
CCG AGT GTG AGC AGA CCC CCT GGT GAA GGG TGA ATC GAC AGG TAC ACA

               (-10)           +1
CAG CCG CCA TAC ACT TCG CTT CAT GCC CTT ACG GGG GGC GGC CAA CCC

AGA AGG AGA TTC TCA ATG ACG TTG TCA AGC CGC CGC GGT AGT GGT TGC
     SD

GGG GTG GTA GAC AGC GTG GTC GCG CAG CAT GGC CCA CAG GAC GTT GAG

GCG GCG GCG GGC CAG GGC GAG GAC GGC TTG GGT GTG GCG TTT TCC TTC

GGT GCG TTT TCG GTC GTA GTA GGT GCG CGA GGA GGG GTC GGT GCG GAT

GCT GAC CAA GGC CGA CAG GTA GCA GGC GCG CAG CAG GCG CCG GTC GTA

GCG TCG GGG GCG TTT GAG GTT TCC GCT GAT GCG GCC GGA ATC TCG TGG

TAC CGG CGC CAG GCC GGC GAC GCC GGC GAG GCG GTC GGC GGA GGC GAA

TGC GGC CAT GTC CCC GCC GGT GGC GGC GAG GAA CTC AGC GCC CAG GAT

GAC GCC GAA TCC GGG CAT GCT CAG GAT GAT TTC GGC GTG GCG GTG GCG

GCG AAA TCG CTC CTC GAT CAT CGC GTC GGT GTC GCC GAT TTC GGT GTC

GAG GGC CAT CAC CTC CTT GGC CAG GCG GGC CAC CAC AGT GGC CGC CAG

TTG TTG GCC GCG CAC GAT GCT GTG TTG GGC GTT AGC GGC CTG CA
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or

(b) a sequence which hybridizes with the sequence complementary to sequence (a).

2. A nucleotide sequence according to claim 1, characterized in that it comprises a sequence selected from the following:

a) the following nucleotide sequence or any part thereof containing the transcription initiation site (position +1), and elements necessary for the recognition and binding of the RNA polymerases of a particular host cell, which will be transformed by this sequence:

GAT CCC GTG ACA AGG CCG AAG AGC CCG CGA CCG TGC GGT CGT
CGA CGA CCG AGT GTG AGC AGA CCC CCT GGT GAA GGG TGA ATC

+1
GAC AGG TAC ACA CAG CCG CCA TAC ACT TCG CTT CAT GCC CTT
ACG GGG GGC GGC CAA CCC AGA AGG AGA TTC TCA

or

b) a sequence which hybridizes with the sequence complementary to sequence a).

3. A nucleotide sequence according to claim 2 in which the elements necessary for the recognition and binding of the RNA polymerases comprise the sequence [TAC ACT] at position -10 relative to the initiation site for transcription and the sequence [TC GAC A] at position -35 relative to that site.

4. A nucleotide sequence according to any one of claims 1 to 3, which in use enables the expression, in a host cell, of a given nucleotide sequence under its control, characterized in that it comprises a sequence selected from:

a) the following nucleotide sequence:
TC GAC AGG TAC ACA CAG CCG CCA TAC ACT TCG CTT CA

b) a sequence which hybridizes with the sequence complementary to sequence a),

or

c) any part of sequence a) or b) involved in the transcription of a nucleic acid.

5. A nucleotide sequence according to any one of claims 1 to 3, characterized in that at least the sequence comprised between positions +2 and +41 in relation to the transcription initiation site is replaced in whole or in part by a sequence that is exogenous in relation to the naturally occurring sequence downstream of the sequence according to claim 4; this exogenous sequence comprising a Shine Dalgarno sequence.

6. A recombinant nucleotide sequence, characterized in that it comprises a nucleotide sequence according to any one of claims 1 to 5 and at least one nucleic acid sequence whose cloning and/or expression is desired in a given host cell, under the control of said nucleotide sequence.

7. A recombinant sequence according to claim 6, characterized in that at least one nucleic acid sequence to be expressed is placed under the control of the nucleotide sequence according to any one of claims 1 to 5, each said nucleic acid sequence coding for an immunogenic peptide or polypeptide or a peptide or polypeptide capable of being rendered immunogenic.

8. A recombinant sequence according to either claim 6 or 7, characterized in that the nucleic acid sequence that is to be expressed codes for a peptide or polypeptide of an HIV retrovirus.

9. A recombinant sequence according to claim 8 wherein the nucleic acid sequence to be expressed codes for an envelope peptide or polypeptide of HIV-1 or HIV-2.

10. A recombinant sequence according to claim 8 wherein the nucleic acid sequence to be expressed codes for a Nef peptide or polypeptide of HIV-1 or HIV-2.

11. A recombinant sequence according to either claim 6 or claim 7, characterized in that the nucleic acid sequence to be expressed is a mycobacterial coding sequence.

12. A recombinant sequence according to claim 11 wherein the nucleic acid sequence to be expressed codes for protein(s) involved in mycobacterial virulence.

13. A recombinant sequence according to claim 11 wherein the nucleic acid sequence to be expressed codes for a mycobacterial protective antigen.

14. A recombinant sequence according to any one of claims 6 to 13, characterized in that the nucleic acid sequence to be expressed codes for one or more specific epitopes.

15. A recombinant nucleotide sequence according to any one of claims 6 to 14, characterized in that the nucleotide sequence according to any one of claims 1 to 5 is in phase, upstream of the nucleic acid sequence that is to be expressed.

16. A recombinant nucleic acid sequence according to any one of claims 6 to 15, characterized in that the nucleotide sequence according to any one of claims 1 to 5 and the nucleic acid sequence or sequence(s) to be expressed constitute an operon fusion.

17. A recombinant nucleotide sequence according to any one of claims 6 to 15, characterized in that the nucleotide sequence according to any one of claims 1 to 5 and the nucleic acid sequence of sequences to be expressed constitute a gene fusion.

18. The use of a sequence according to any one of claims 1 to 5 for the cloning and/or expression of nucleic acid sequences in a host cell other than an Mptb host cell.

19. The use as defined in claim 18 wherein the host is an actinomycetes host.

20. The use as defined in claim 18 wherein the host cell is M.bovis.

21. The use as defined in claim 20 wherein the host cell is M.bovis BCG.

22. The use as defined in claim 18 wherein the host is a gram-negative bacteria.

23. The use as defined in claim 22 wherein the host is E. coli.

24. The use as defined in claim 18 wherein the host is a gram-positive bacteria.

25. The use as defined in claim 24 wherein the host is B. subtilis.

26. A cloning and/or expression vector of the integrative type or replicative type, characterized in that it comprises, in a site not essential for its integration or replication, a nucleotide sequence according to any one of claims 1 to 5.

27. A cloning and/or expression vector of the integrative type or replicative type, characterized in that it is modified, at a site that is not essential for its integration or its replication, by a recombinant nucleotide sequence according to any one of claims 6 to 17.

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28. A vector according to claim 27, characterized in that it contains, in addition, a part of the Mptb sequence designated ORF2, downstream of the nucleotide sequence according to any one of claims 1 to 5.

29. A vector according to any one of claims 26 to28, characterized in that it is a plasmid, a transposon, or a phage.

30. A vector according to claim 29, characterized in that it is contained in the E. coli strain deposited in the "Collection Nationale des Microorganismes" on 23 October 1991 as no. I-1157.

31. A vector according to any one of claims 26 to 30, characterized in that the nucleotide sequence according to any one of claims 1 to 5 is upstream of the nucleic acid sequence or sequences that it controls.

32. A vector according to any one of claims 26 to 30, characterized in that the nucleotide sequence according to any one of claims 1 to 5 is downstream of the nucleic acid sequence or sequences that it controls.

33. A vector according to any one of claims 26 to 32, characterized in that it includes a marker of expression.

34. A vector according to any one of claims 26 to 33, characterized in that it comprises, in addition, elements regulating the expression in a given host cell of the nucleic acid sequence or sequences that it contains.

35. A recombinant host cell, characterized in that it is transformed by a recombinant nucleotide sequence according to any one of claims 6 to 17 or by a vector according to any one of claims 26 to 34, in conditions enabling the expression of the nucleic acid sequence or sequences contained in the recombinant sequence or in the vector.

36. A recombinant host cell according to claim 35, characterized in that it enables the presentation at its surface, or indeed the exportation, secretion, or excretion, of the expression product of the nucleic acid sequence or sequences that it contains.

37. A recombinant host cell according to either claim 35 or claim 36, characterized in that it is a cell of an actinomycetes strain.

38. A recombinant host cell according to claim 37 which is a cell of a nonvirulent

39. A recombinant host cell according to claim 38 which is a cell of an M. bovis BCG strain.

40. A recombinant host cell according to either claim 35 or claim 36, characterized in that it is a gram-negative bacterium.

41. A recombinant host cell according to claim 40 wherein the gram-negative bacterium is a strain of E. coli.

42. A recombinant host cell according to either claim 35 or claim 36, characterized in that it is a gram-positive bacterium.

43. A recombinant host cell according to claim 42 wherein the gram-positive bacterium is B. subtilis.

44. A recombinant host cell according to claim 42 wherein the gram-positive bacterium is a strain of Streptomyces.

45. An immunogenic composition characterized in that it comprises a recombinant host cell according to either claim 35 or claim 36 in sufficient quantity to effect the production of antibodies or to contribute to the production of antibodies in an animal host to which it is administered, and/or a cell-mediated immune response.

46. An immunogenic composition according to claim 45 which comprises a recombinant host cell according to claim 35 or claim 36 in sufficient quantity to effect the production of protective antibodies in an animal host to which it is administered.

47. An immunogenic composition according to claim 45 which comprises a recombinant host cell according to claim 35 or claim 36 in sufficient quantity to effect a TCL response in an animal host to which it is administered.

48. An immunogenic composition which comprises a recombinant host cell according to claim 35 or claim 36 in a quantity sufficient to induce an immunologically protective response in a host to which said composition is administered.

49. A nucleotide sequence as defined in claim 1 substantially as herein described with reference to any example thereof or to the accompanying drawings.

50. A recombinant nucleotide sequence as defined in claim 6 substantially as herein described with reference to any example thereof or to the accompanying drawings.

51. The use of a sequence as defined in claim 18 substantially as herein described with reference to any example thereof or to the accompanying drawings.

52. A cloning and/or expression vector as defined in claim 26 substantially as herein described with reference to any example thereof or to the accompanying drawings.

53. A cloning and/or expression vector as defined in claim 27 substantially as herein described with reference to any example thereof or to the accompanying drawings.

54. A recombinant host cell as defined in claim 35 or claim 36 substantially as herein described with reference to any example thereof.

55. An immunogenic composition as defined in claim 45 substantially as herein described with reference to any example thereof or to the accompanying drawings.

56. An immunogenic composition as defined in claim 48 substantially as herein described with reference to any example thereof or to the accompanying drawings.

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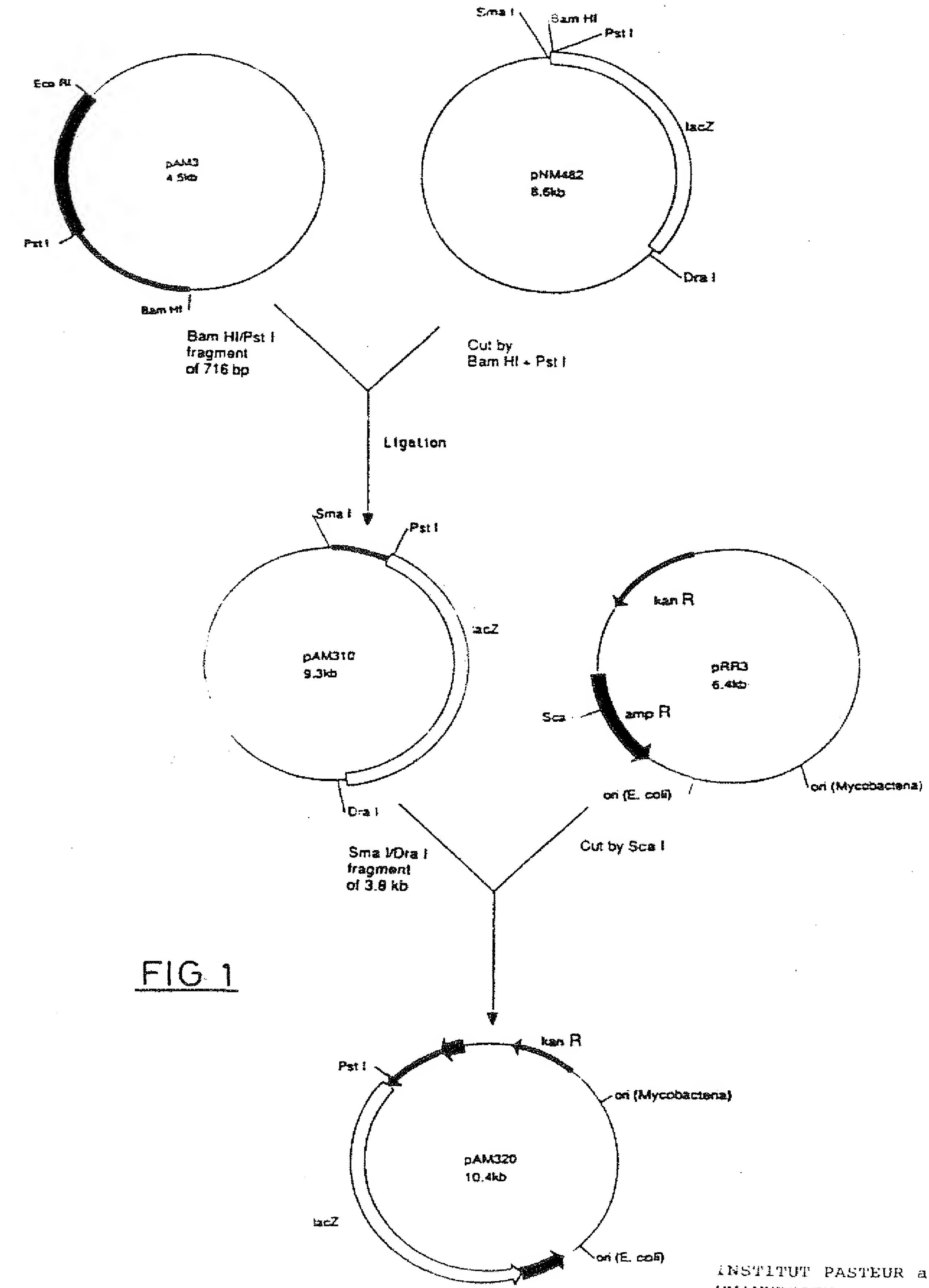

FIG 1

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1
GAT CCC GTG ACA AGG CCG AAG AGC CCG CGA CCG TGC GGT CGT CGA CGA

                                              (-35)
CCG AGT GTG AGC AGA CCC CCT GGT GAA GGG TGA ATC GAC AGG TAC ACA

  101      (-10)          +1
CAG CCG CCA TAC ACT TCG CTT CAT GCC CTT ACG GGG GGC GGC CAA CCC


AGA AGG AGA TTC TCA ATG ACG TTG TCA AGC CGC CGC GGT AGT GGT TGC
     SD                 Met Thr Leu Ser Ser Arg Arg Gly Ser Gly Cys
        201
GGG GTG GTA GAC AGC GTG GTC GCG CAG CAT GGC CCA CAG GAC GTT GAG
Gly Val Val Asp Ser Val Val Ala Gln His Gly Pro Gln Asp Val Glu

GCG GCG GCG GGC CAG GGC GAG GAC GGC TTG GGT GTG GCG TTT TCC TTC
Ala Ala Ala Gly Gln Gly Glu Asp Gly Leu Gly Val Ala Phe Ser Phe
              301
GGT GCG TTT TCG GTC GTA GTA GGT GCG CGA GGA GGG GTC GGT GCG GAT
Gly Ala Phe Ser Val Val Val Gly Ala Arg Gly Gly Val Gly Ala Asp

GCT GAC CAA GGC CGA CAG GTA GCA GGC GCG CAG CAG GCG CCG GTC GTA
Ala Asp Gln Gly Arg Gln Val Ala Gly Ala Gln Gln Ala Pro Val Val
                     401
GCG TCG GGG GCG TTT GAG GTT TCC GCT GAT GCG GCC GGA ATC TCG TGG
Ala Ser Gly Ala Pha Glu Val Ser Ala Asp Ala Ala Gly Ile Ser Trp

TAC CGG CGC CAG GCC GGC GAC GCC GGC GAG GCG GTC GGC GGA GGC GAA
Tyr Arg Arg Gln Ala Gly Asp Ala Gly Glu Ala Val Gly Gly Gly Glu
                        501
TGC GGC CAT GTC CCC GCC GGT GGC GGC GAG GAA CTC AGC GCC CAG GAT
Cys Gly His Val Pro ALa Gly Gly Gly Glu Glu Leu Ser Ala Gln Asp

GAC GCC GAA TCC GGG CAT GCT CAG GAT GAT TTC GGC GTG GCG GTG GCG
Asp Ala Glu Ser Gly His Ala Gln Asp Asp Phe Gly Val Ala Val Ala
                                601
GCG AAA TCG CTC CTC GAT CAT CGC GTC GGT GTC GCC GAT TTC GGT GTC
Ala Lys Ser Leu Leu Asp His Arg Val Gly Val Ala Asp Phe Gly Val

GAG GGC CAT CAC CTC CTT GGC CAG GCG GGC CAC CAC AGT GGC CGC CAG
Glu Gly His His Leu Leu Gly Gln Ala Gly His His Ser Gly Arg Gln
                                    701                 716
TTG TTG GCC GGG CAC GAT GCT GTG TTG GGC GTT AGC GGC CTG CA
Leu Leu Ala Gly His Asp Ala Val Leu Gly Val Ser Gly Leu
```

FIG 2

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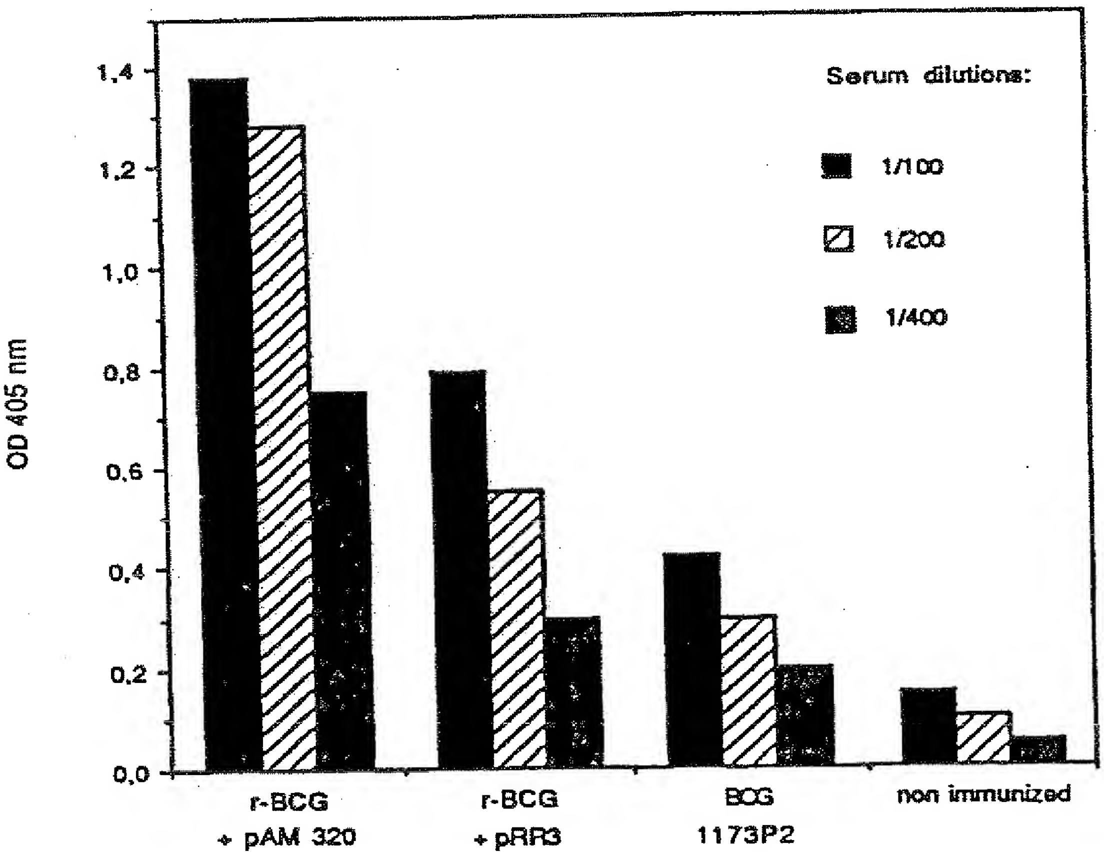

FIG 3

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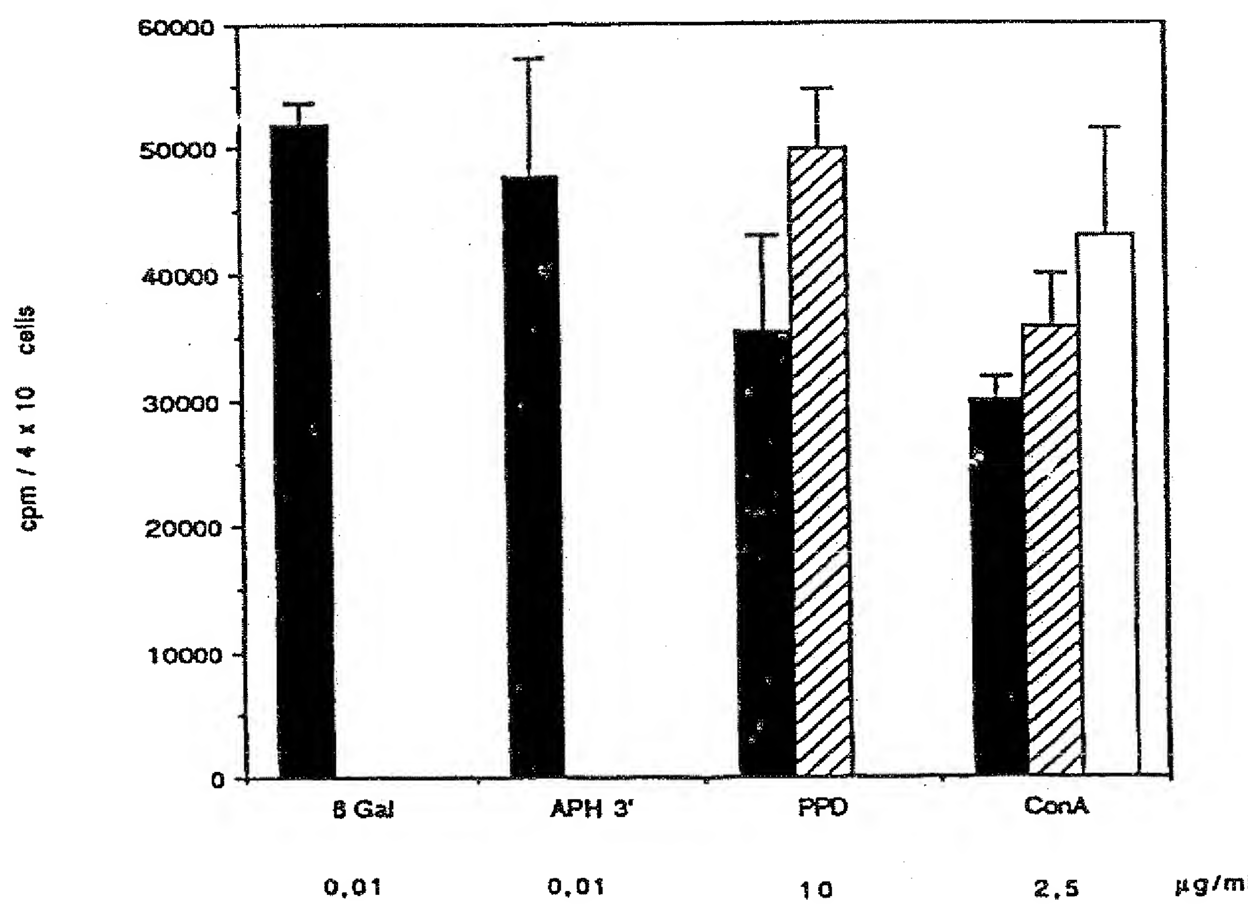

FIG 4

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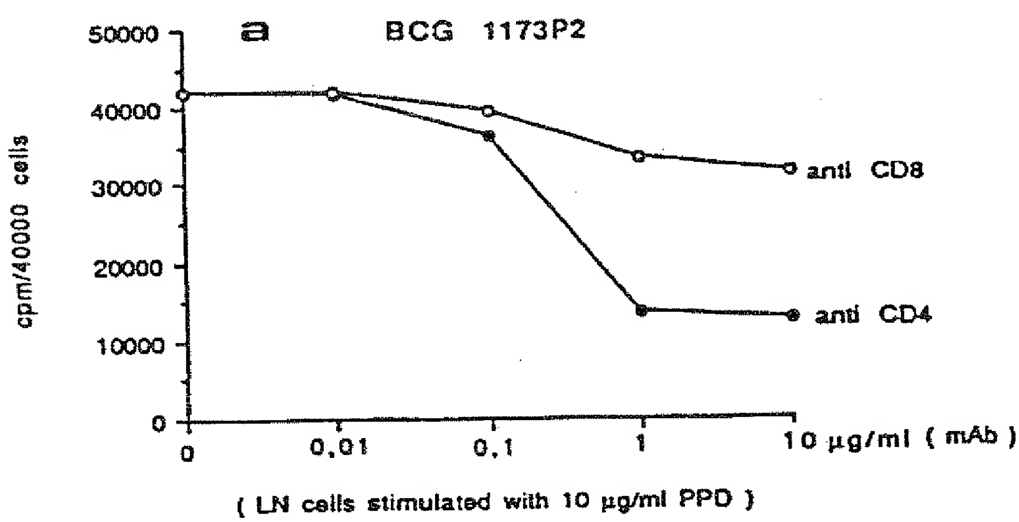

FIG 5 (a)

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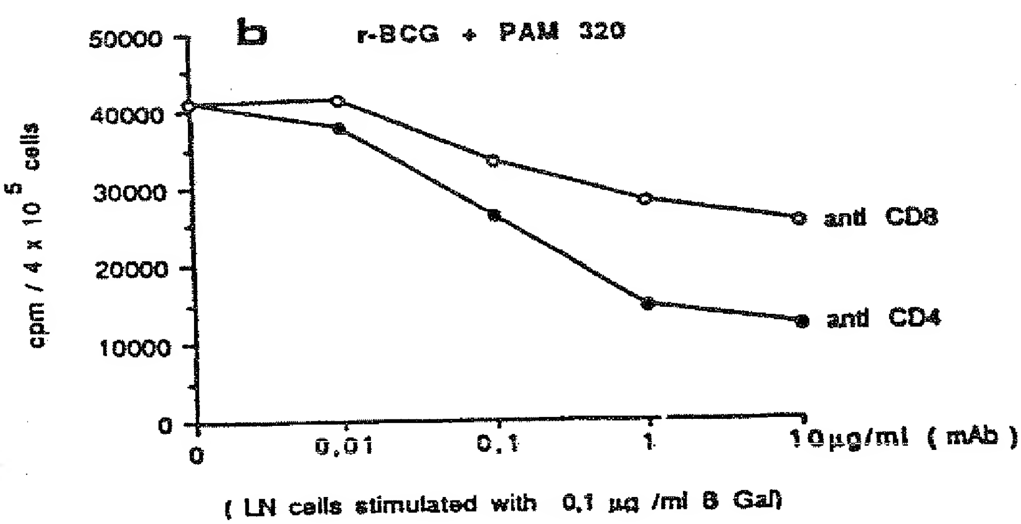

FIG 5(b)

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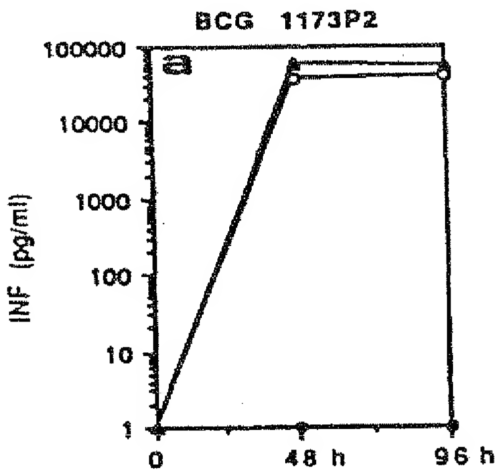

FIG 6(a)

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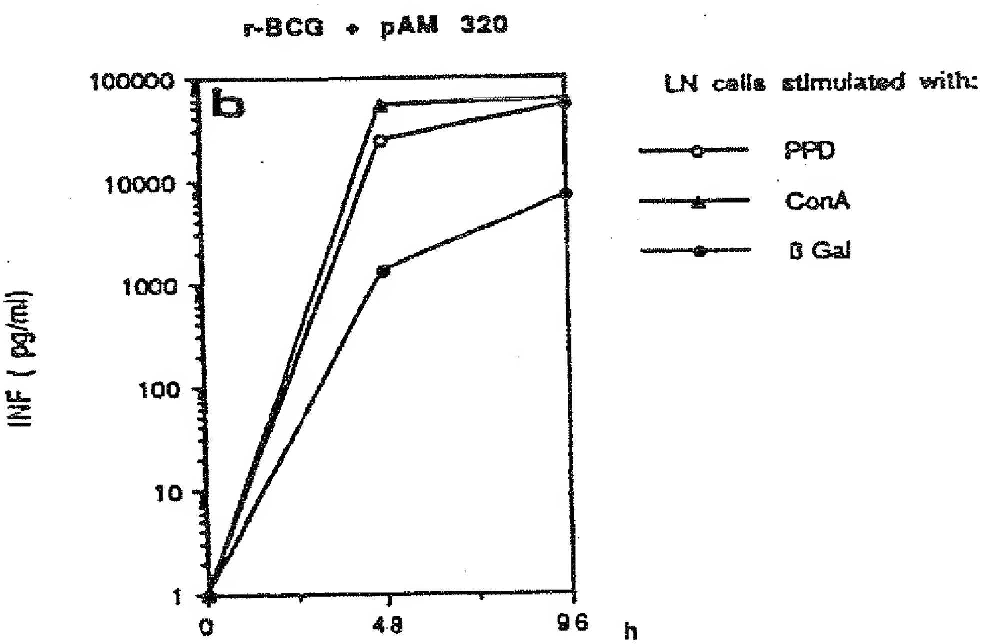

FIG 6(b)

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Primer No. 1
5' CCCTCTAGAATTCCGTGACAAGGCCGAAGAGCCCGCGA 3'

Primer No. 2
5' AACATATGAGATCTTCTCCTTCTGGGTTGGCCGCCCC 3'

Position of primers in fragment to be amplified.

```
CCCTCTAGAATTCCGTGACAAGGCCGAAGAGCCCGCGA 3'
        1        11        21        31        41        51
        GATCCCGTGACAAGGCCGAAGAGCCCGCGACCGTGCGGTCGTCGACGACCGAGTGTG

        61       71        81        91       101       111
        AGCAGACCCCCTGGTGAAGGGTGAATCGACAGGTACACACAGCCGCCATACACTTCG

        121       131       141       151        161
        CTTCATGCCCTTACGGGGGGCGGCCAACCCAGAAGGAGATTCTCA ATG ACG TTG
                                             SD         Met Thr Leu
                    3'CCCCGCCGGTTGGGTCTTCCTCTTCTAGA GTA TAC AA

        171
        Ser Ser Arg Arg
        TCA AGC CGG CGC
```

FIG 7

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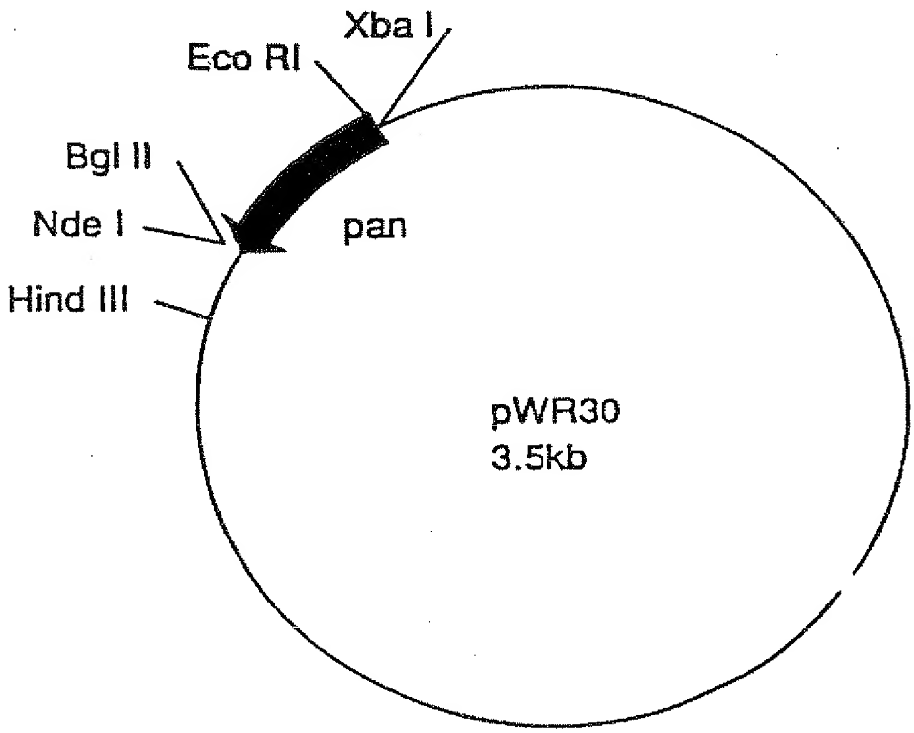

FIG 8

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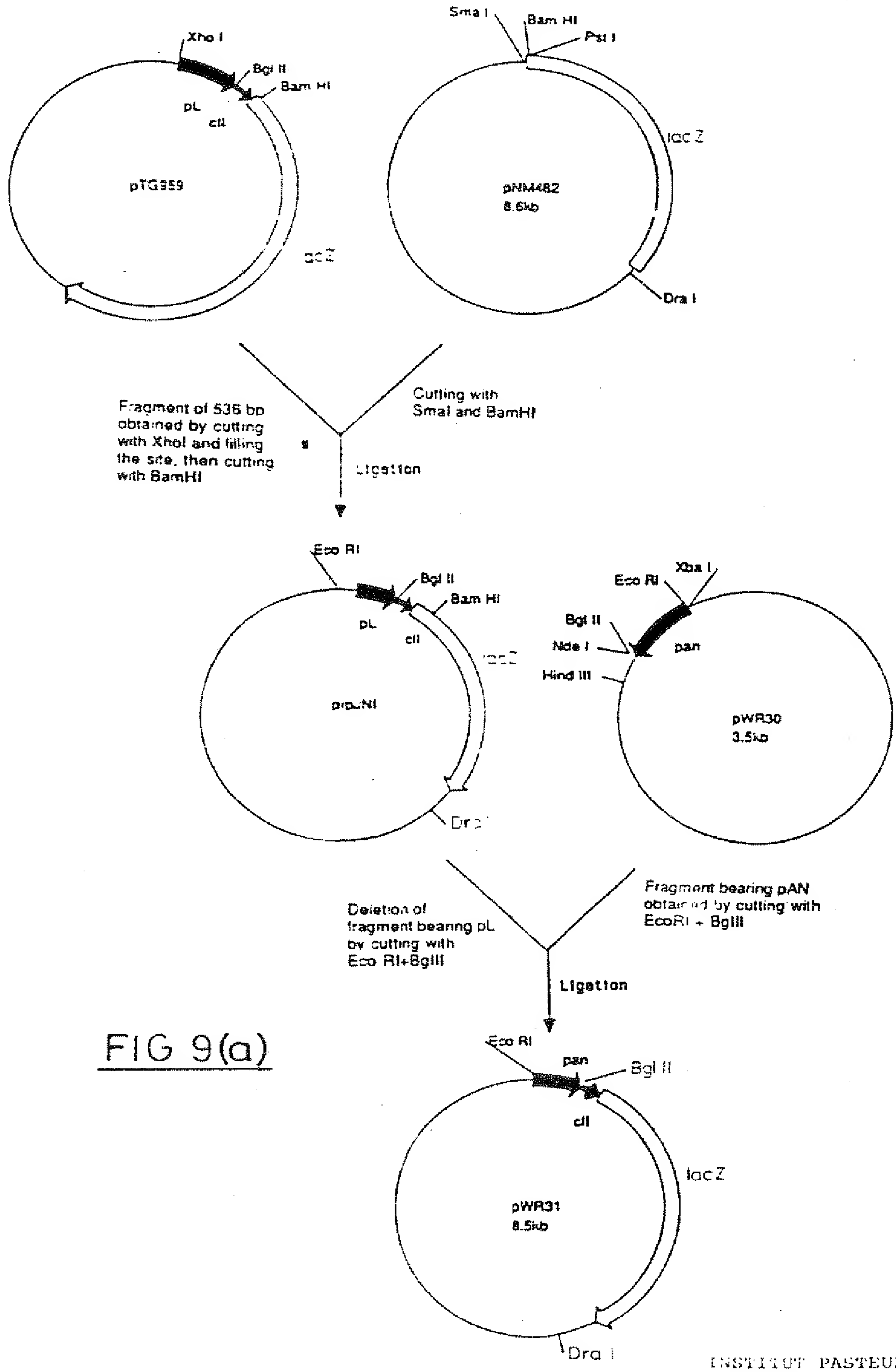

FIG 9(a)

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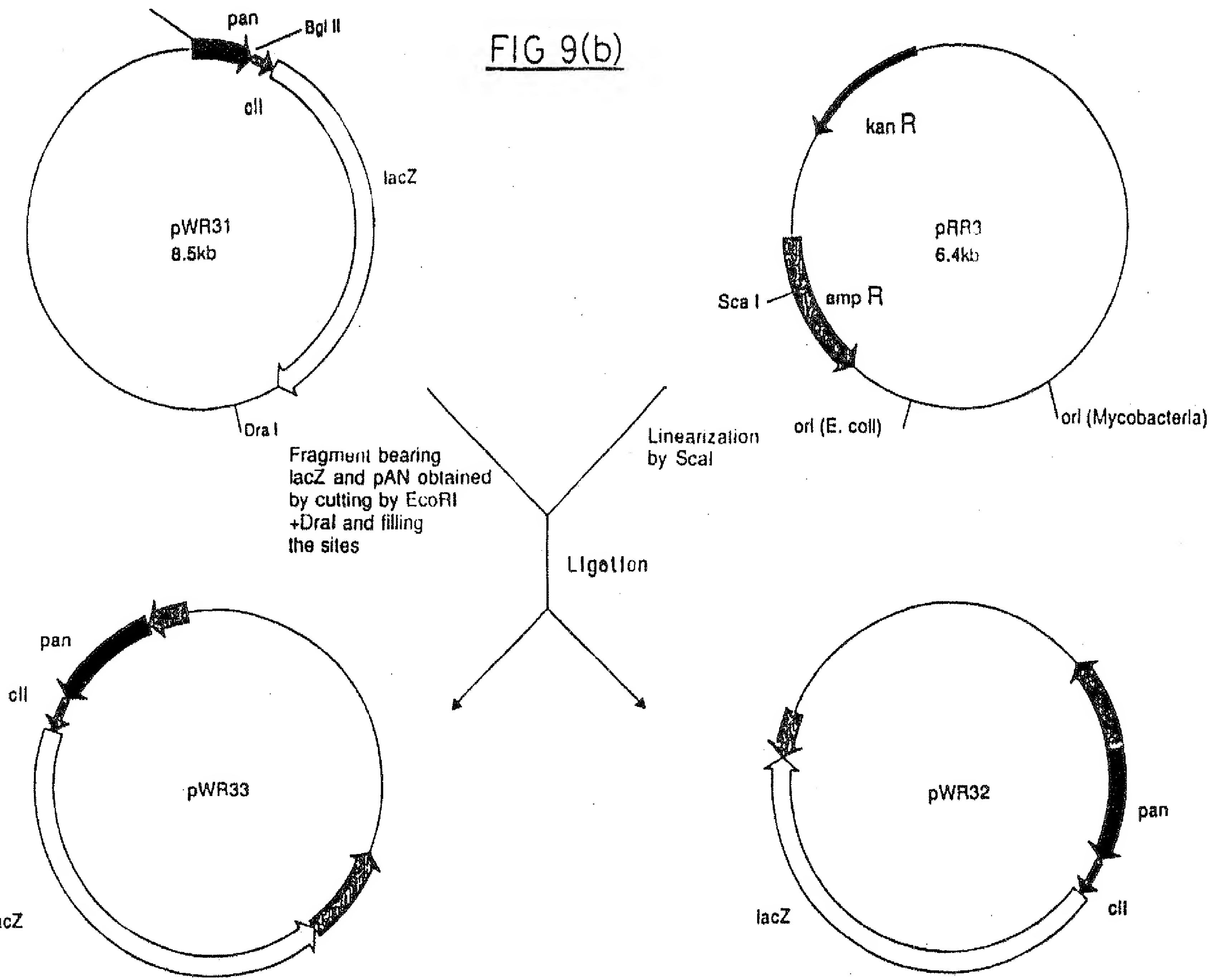
FIG 9(b)



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```
1                   11                  21                  31                  41                  51                  61                  71                  81
GAT CCC GTG ACA AGG CCG AAG AGC CCG CGA CCG TGC GGT CGT CGA CGA CCG AGT GTG AGC AGA CCC CCT GGT GAA GGG TGA ATC GAC AGG

91                  101                 111                 121                 131                 141                 151                 161                 171
TAC ACA CAG CCG CCA TAC ACT TCG CTT CAT GCC CTT ACG GGG GGC GGC CAA CCC AGA AGG AGA TTC TCA ATG ACG TTG TCA AGC CGC CGC
                                                                               SD                      Met Thr Leu Ser Ser Arg Arg

181                 191                 201                 211                 221                 231                 241                 251                 261
GGT AGT GGT TGC GGG GTG GTA GAC AGC GTG GTC GCG CAG CAT GGC CCA CAG GAC GTT GAG GCG GCG GCG GGC CAG GGC GAG GAC GGC TTG
Gly Ser Gly Cys Gly Val Val Asp Ser Val Val Ala Gln His Gly Pro Gln Asp Val Glu Ala Ala Ala Gly Gln Gly Glu Asp Gly Leu

271                 281                 291                 301                 311                 321                 331                 341                 351
GGT GTC GCG TTT TCC TTC GGT GCG TTT TCG GTC GTA GTA GGT GCG CGA GGA GGG GTC GGT GCG GAT GCT GAC CAA GGC CGA CAG GTA GCA
Gly Val Ala Phe Ser Phe Gly Ala Phe Ser Val Val Val Gly Ala Arg Gly Gly Val Gly Ala Asp Ala Asp Gln Gly Arg Gln Val Ala

361                 371                 381                 391                 401                 411                 421                 431                 441
GGC GCG CAG CAG GCG CCG GTC GTA GCG TCG GGG GCG TTT GAG GTT TCC GCT GAT GCG GCC GGA ATC TCG TGG TAC CGG CGC CAG GCC GGC
Gly Ala Gln Gln Ala Pro Val Val Ala Ser Gly Ala Phe Glu Val Ser Ala Asp Ala Ala Gly Ile Ser Trp Tyr Arg Arg Gln Ala Gly

451                 461                 471                 481                 491                 501                 511                 521                 531
GAC GCC GGC GAG GCG GTC GGC GGA GGC GAA TGC GGC CAT GTC CCC GCC GGT GGC GGC GAG GAA CTC AGC GCC CAG GAT GAC GCC GAA TCC
Asp Ala Gly Glu Ala Val Gly Gly Gly Glu Cys Gly His Val Pro Ala Gly Gly Gly Glu Glu Leu Ser Ala Gln Asp Asp Ala Glu Ser

541                 551                 561                 571                 581                 591                 601                 611                 621
GGG CAT GCT CAG GAT GAT TTC GGC GTG GCG GTG GCG GCG AAA TCG CTC CTC GAT CAT CGC GTC GGT GTC GCC GAT TTC GGT GTC GAG GGC
Gly His Ala Gln Asp Asp Phe Gly Val Ala Val Ala Ala Lys Ser Leu Leu Asp His Arg Val Gly Val Ala Asp Phe Gly Val Glu Gly

631                 641                 651                 661                 671                 681                 691                 701                 711
CAT CAC CTC CTT GGC CAG GCG GGC CAC CAC AGT GGC CGC CAG TTG TTG GCC GGG CAC GAT GCT GTG TTG GGC GTT AGC GGC CTG CAG CGC
His His Leu Leu Gly Gln Ala Gly His His Ser Gly Arg Gln Leu Leu Ala Gly His Asp Ala Val Leu Gly Val Ser Gly Leu Gln Arg

721                 731                 741                 751                 761                 771                 781                 791                 801
GGT GGC TGC GAC GGT ATC GGC GTT GCG GGC CTT GCG TTT ACG CAA GAA GCG GGC TAC TCG AGC GCC ACC GGC GCT GCG CAG CGC GTC GGG
Gly Gly Cys Asp Gly Ile Gly Val Ala Gly Leu Ala Phe Thr Gln Glu Arg Gly Tyr Ser Ser Ala Thr Gly Ala Ala Gln Arg Val Gly
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FIG 10(a)

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FIG 10(b)

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811       821       831       841       851       861       871       881       891
AGT TTG GTA GCC AGT AAG CAG GAT CAG CGC GGC ACG GCT CTT GTT GTA GTC GAA GGC GCG TTC CAG CGC CGG AAA GTA TTC CAG CAG CTG
Ser Leu Val Ala Ser Lys Gln Asp Gln Arg Gly Thr Ala Leu Val Val Val Glu Gly Ala Phe Gln Arg Arg Lys Val Phe Gln Gln Leu

901       911       921       931       941       951       961       971       981
GGC GCG CAT TCG GTT GAT CGC CCG GGT CCG ATC AGC CAC CAG ATC GGA ACG TCG GCT GGT CAG GAT GCG CAG CTC GAC TGC GAT GTC ATC
Gly Ala His Ser Val Asp Arg Pro Gly Pro Ile Ser His Gln Ile Gly Thr Ser Ala Gly Gln Asp Ala Gln Leu Asp Cys Asp Val Ile

991       1001      1011      1021      1031      1041      1051      1061      1071
GCC GGC GCG CAG AGG CTG CAA GTC GTG GCG CAT CCG GGC CTG ATC GGC GAT GAT CGC AGC GTC TTT GGC GTC GGT CTT GCC TTC GCC GCG
Ala Gly Ala Gln Arg Leu Gln Val Val Ala His Pro Gly Leu Ile Gly Asp Asp Arg Ser Val Phe Gly Val Gly Leu Ala Phe Ala Ala

1081      1091      1101      1111      1121      1131      1141      1151      1161
GTA ACT ACC CGC GGC GTG ATG GAC CGT GCG CCC GGG AAT ATA AAG CAG CCG CTG CCC GGC AGC GAT GAG CAA GGC GAT CAG CAA CGC GGC
Val Thr Thr Arg Gly Val Met Asp Arg Ala Pro Gly Asn Ile Lys Gln Pro Leu Pro Gly Ser Asp Glu Gln Gly Asp Gln Gln Arg Gly

1171      1181      1191      1201      1211      1221      1231      1241      1251
GCC GCC GGC GTT GAG GTC GAT CGC CCA CGT GAC CTC GCC TCC ATC GGC CAA CGT CGT CAC CGC CGC AAA TCA ACT CCA GCA GCG CGG CCT
Ala Ala Gly Val Glu Val Asp Arg Pro Arg Asp Leu Ala Ser Ile Gly Gln Arg Arg His Arg Arg Lys Ser Thr Pro Ala Ala Arg Pro

1261      1271      1281      1291      1301      1311      1321      1331      1341
CGT CGT TGG CCA CCC GCT GCG AGA GCA ATC GCT GCG CGT CGT CGT TAA TAA CCA TGC AGT AAT GGT CGG CCT TAC CGG CGT CCA CGC CCG
Arg Arg Trp Pro Pro Ala Ala Arg Ala Ile Ala Ala Arg Arg Arg TER TER

1351      1361      1371      1381      1391      1401      1411      1421      1431
CCC AGA CAG GTT GTG CCA CAA CCA CCT CCG TAA CCG TCA TTG TCC AGA TCA ACC CAG CAG ACG ACC ACG CCG ACG TGT CCT TAC ACA GCG

1441      1451      1461      1471      1481      1491      1501      1511      1521
ATC CA  TCG CAT CTC TCA ATT AGC GGT CGA GTC GTC GCG GGA CGC CGG GCG GCC AAT CTC CTT CGG CCA TCC AAC ACA GCA ACC ACA TGA

1531      1541      1551      1561      1571      1581      1591      1601      1611
AAG CCA TAC CCG ACG TCC CTG GGC AAT TCG AAG CCT AAG CCG ACG GCC CCG AAC ACC CTT CAA GAA AGG TAA GGA ATT
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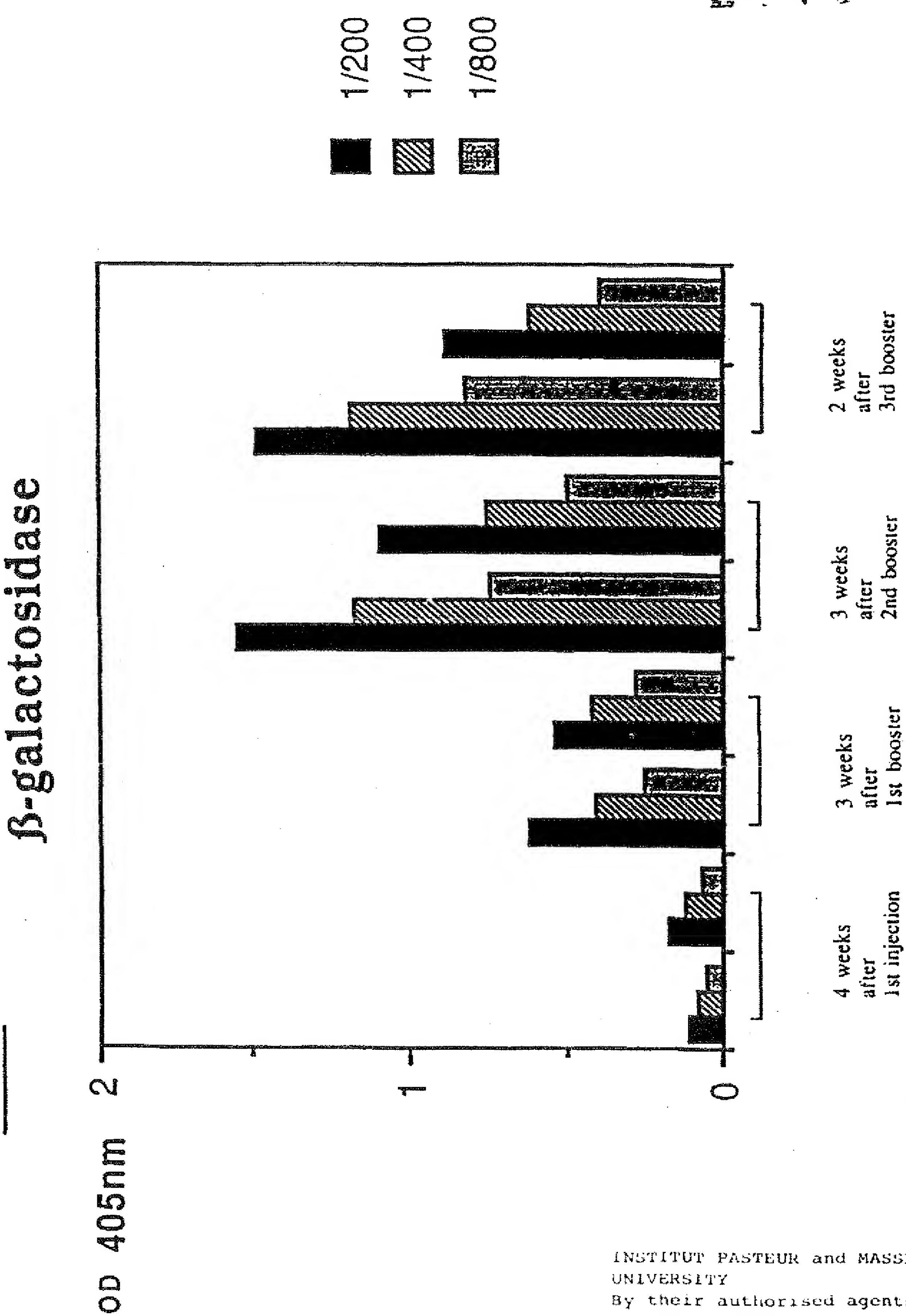
FIG 11



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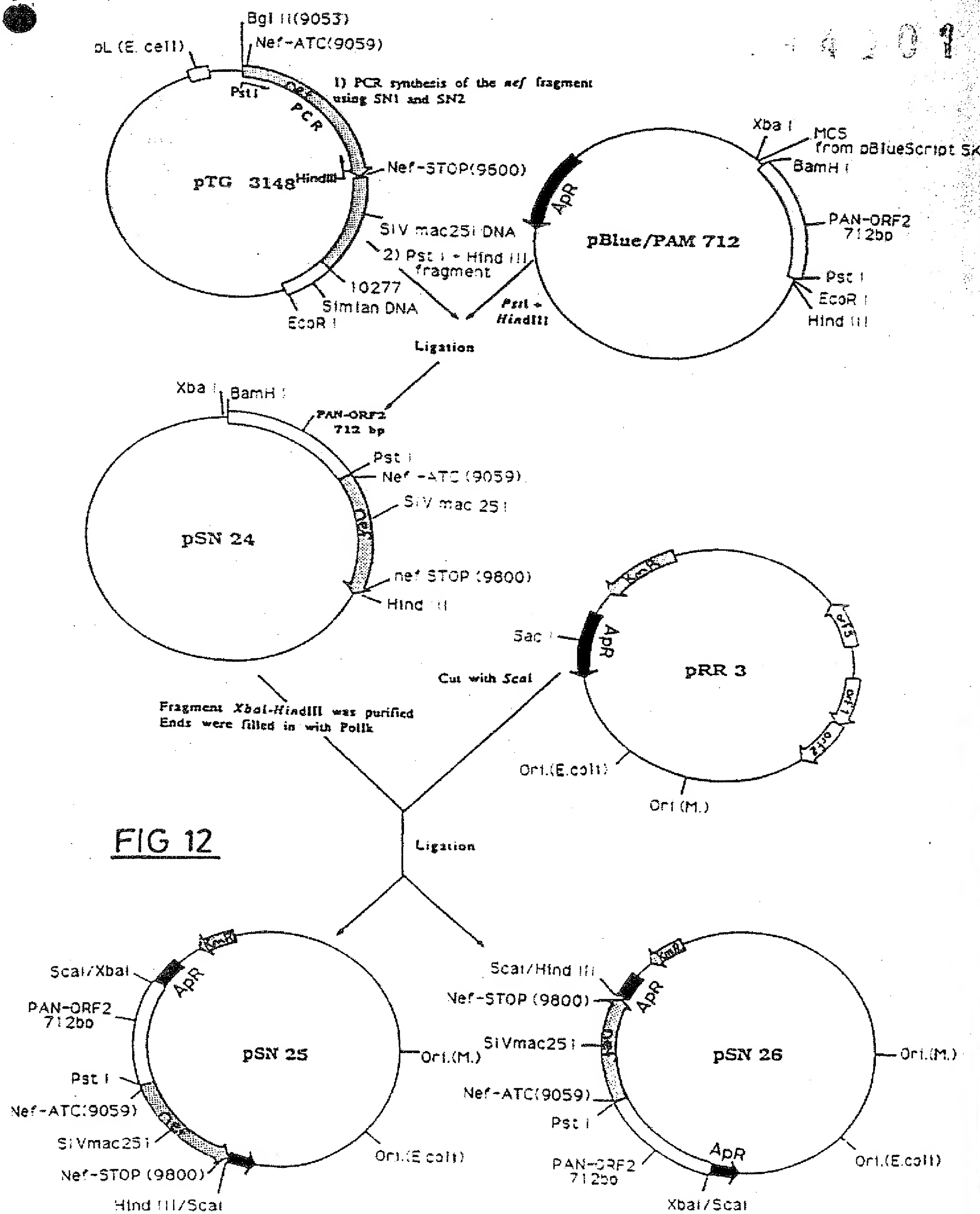

FIG 12

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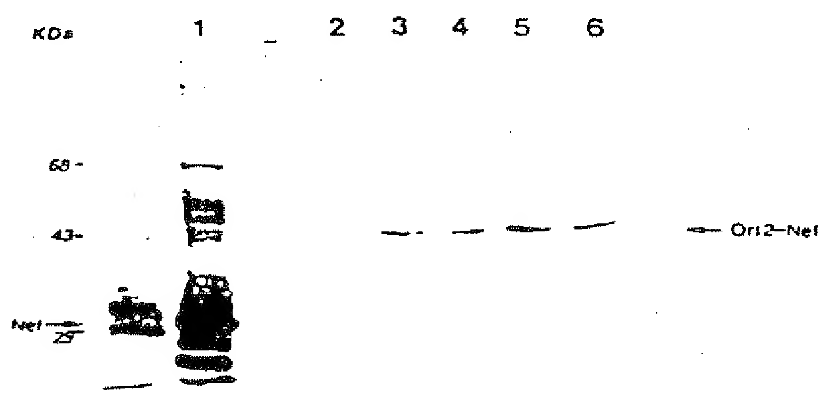

FIG 13

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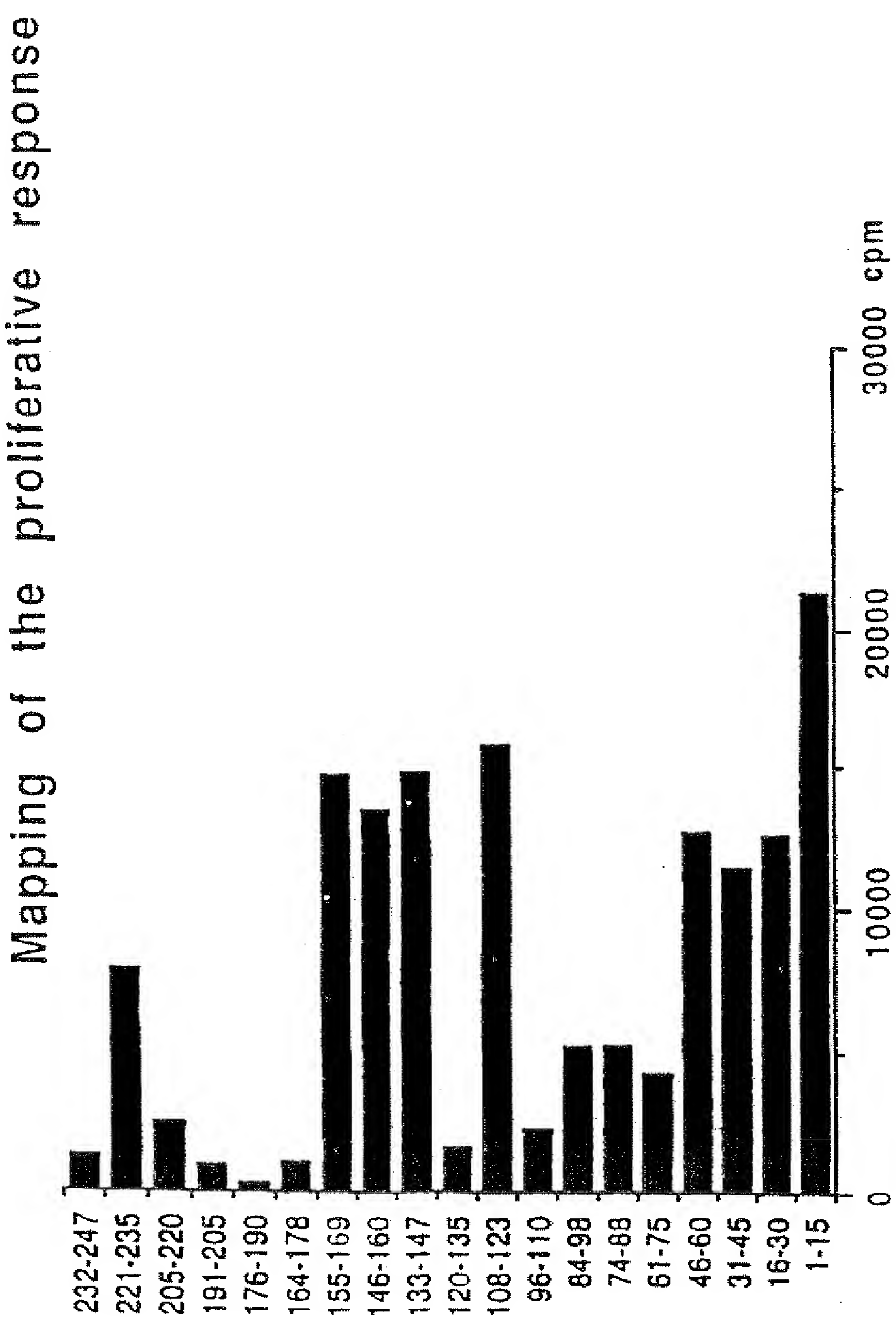

FIG 14

Peptides

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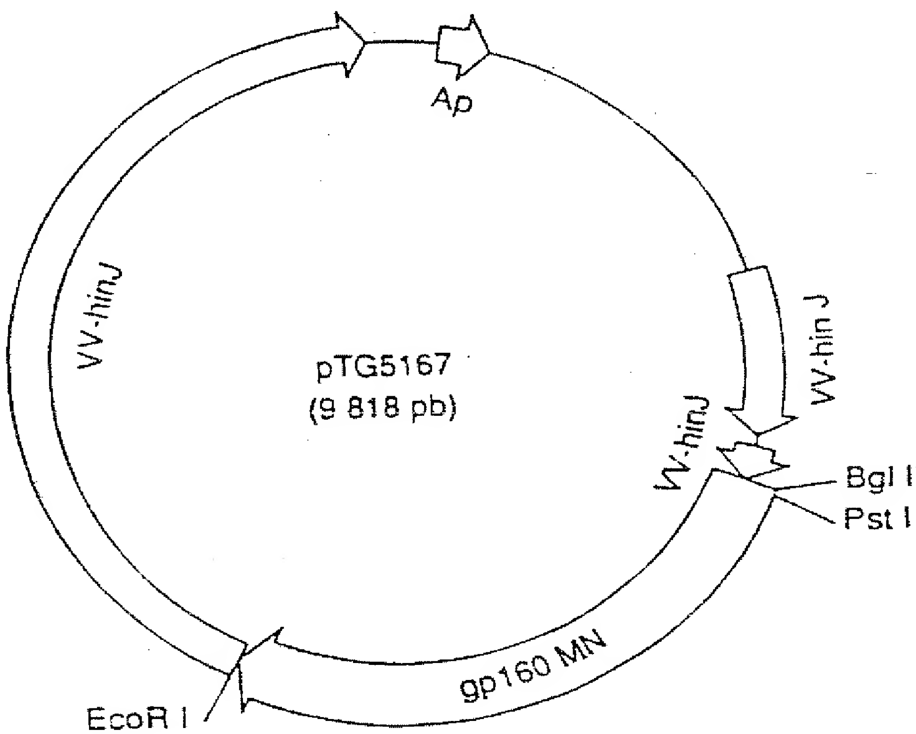

FIG 15

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Construction of plasmids pLA12 and pLA13

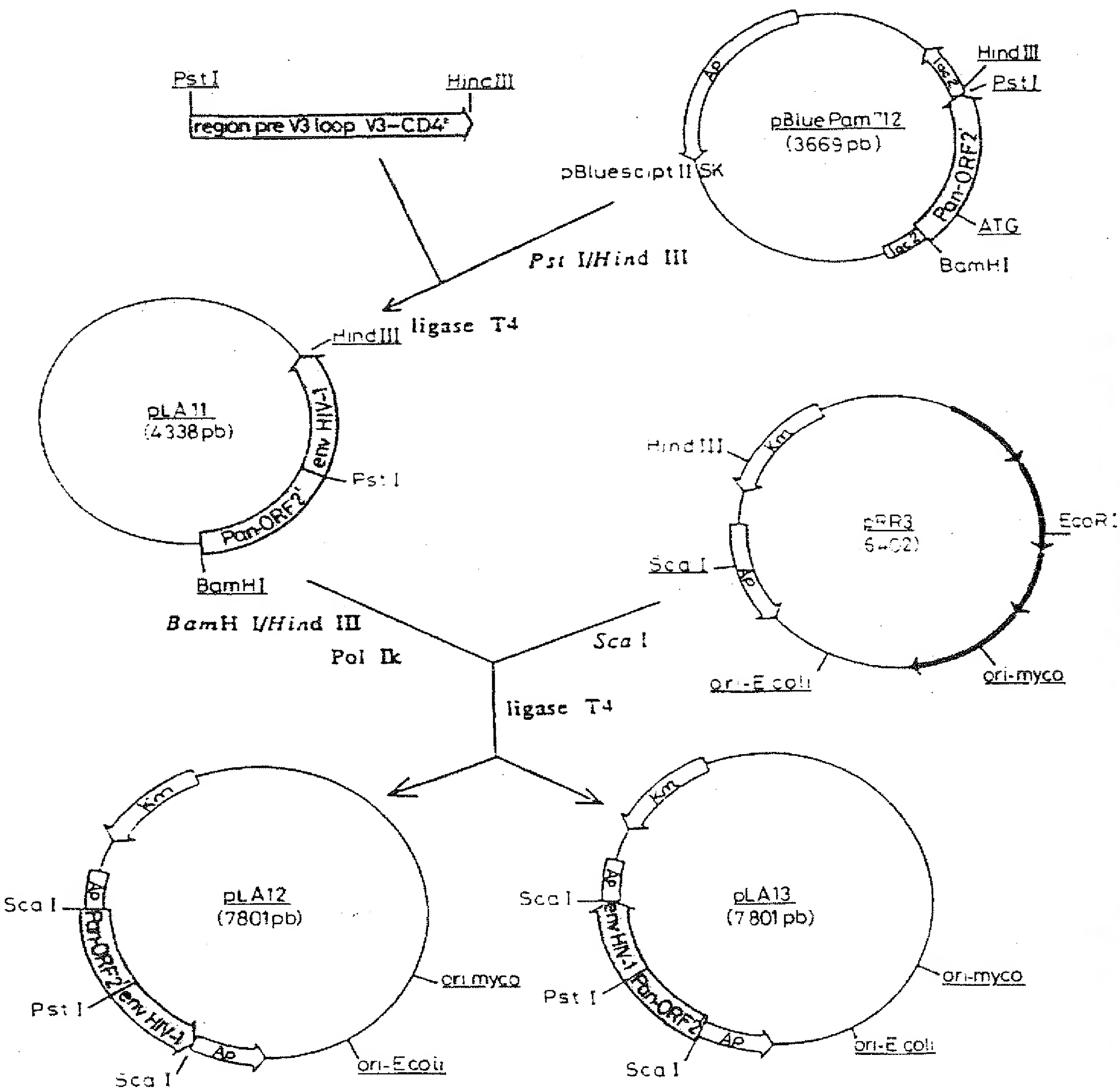

FIG 16

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1. Protein gp120, from HIV-1_{LAI}
2. Standard BCG, cell extract
3. BCG-pLA12, cell extract
4. BCG-pLA13, cell extract
5. BCG-pLA12, culture supernatant
6. BCG-pLA13, culture supernatant

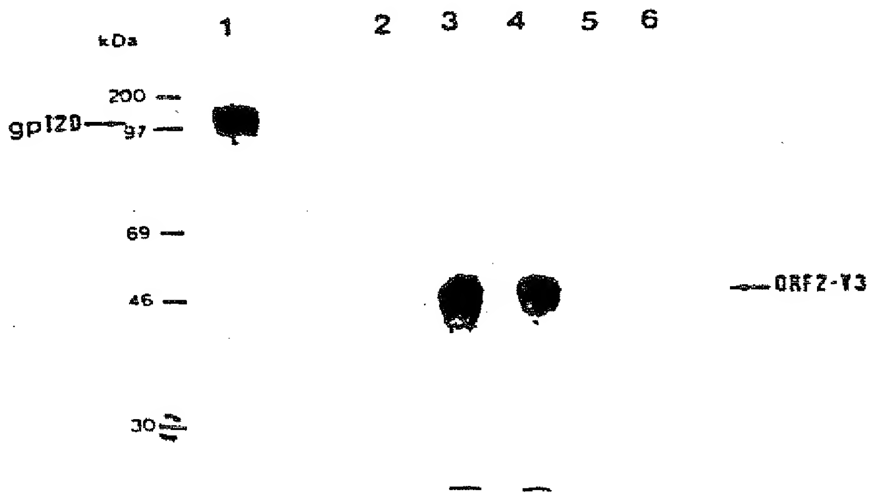

FIG 17

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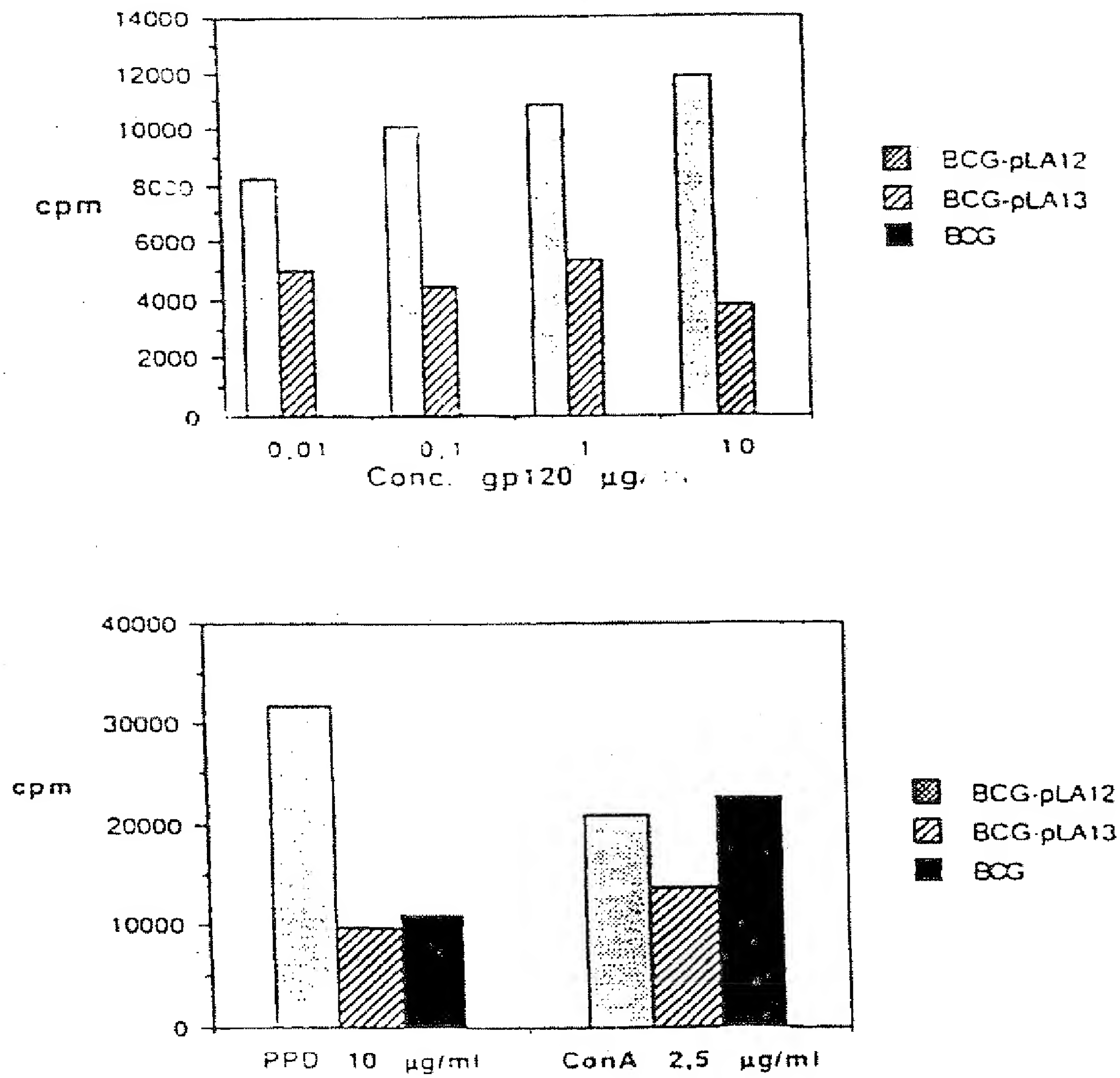

FIG 18

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Per:

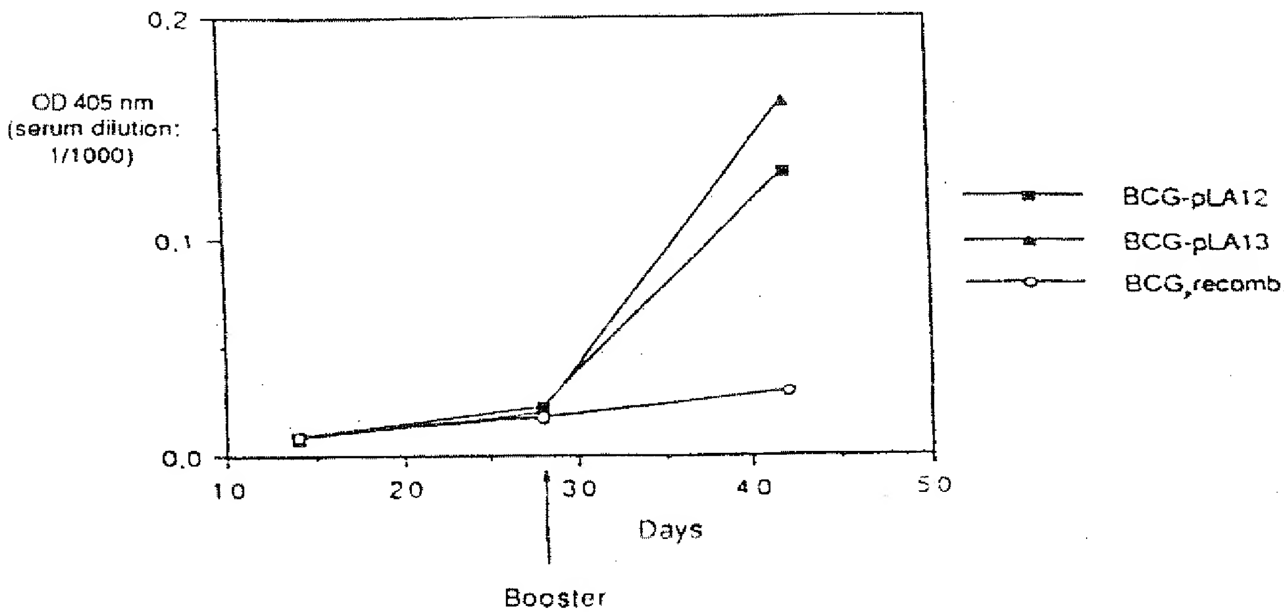

FIG 19

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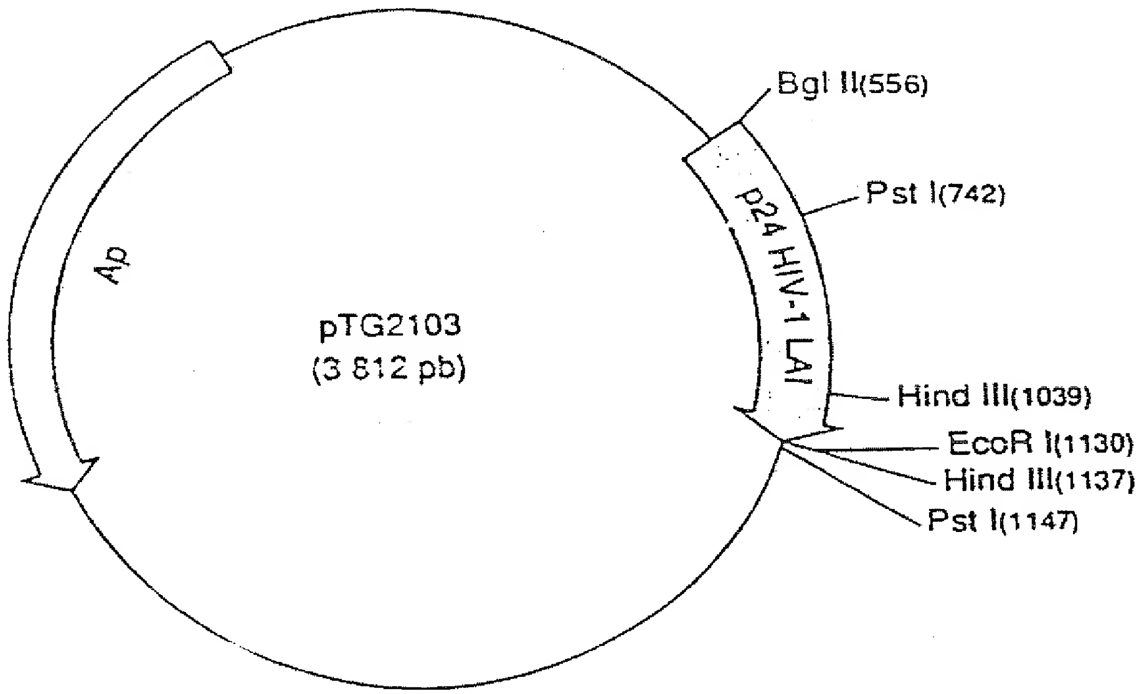

FIG 20

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Per:

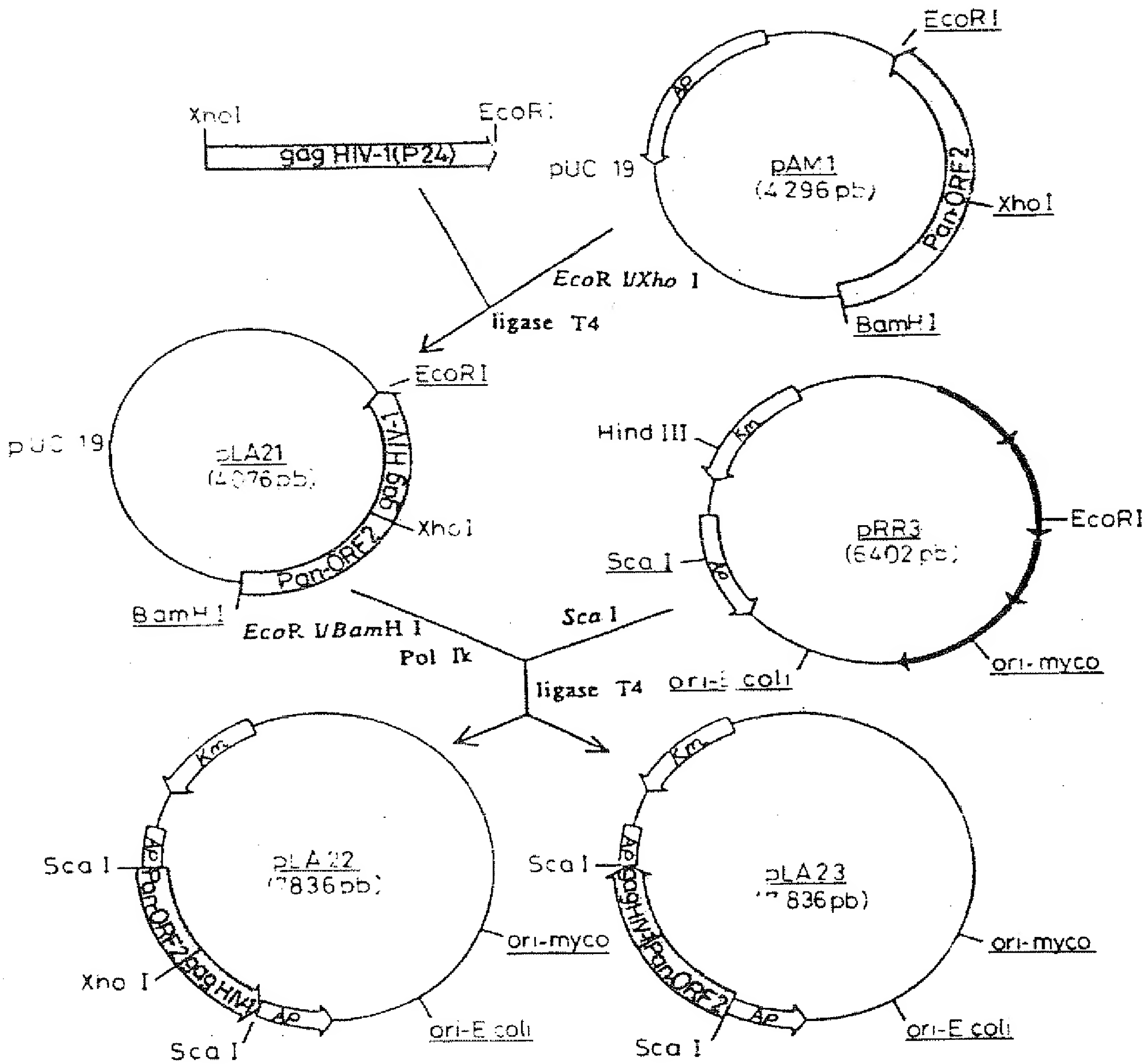

FIG 21

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Per:

1. Protein P24
2. BCG-pRR3, cell extract
3. BCG-pLA22.1, cell extract
4. BCG-pLA22.2, cell extract
5. BCG-pLA23, cell extract
6. BCG-pLA22.1, culture supernatant
7. BCG-pLA22.2, culture supernatant
8. BCG-pLA23, culture supernatant

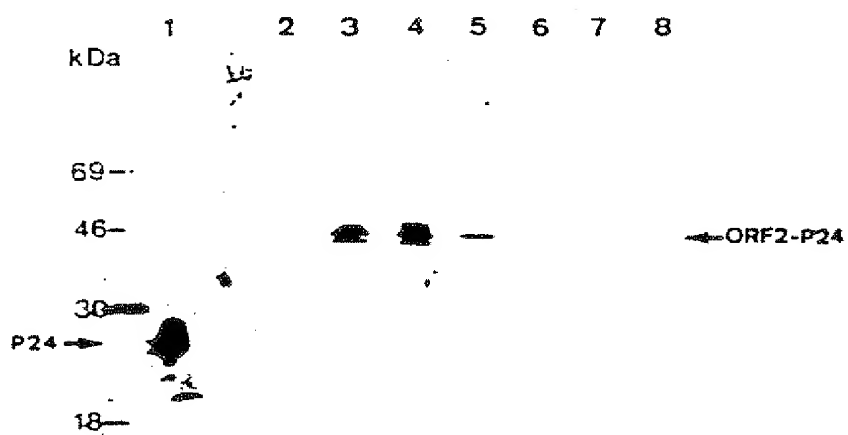

FIG 22

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